Effect of Ethyl Oleate Pheromone on Honey Bee (*Apis mellifera)* Overwintering Physiology

By

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Abstract

In winter, honey bees undergo a transition to a diutinus state, during which time brood rearing declines or stops entirely, and worker bees live for up to 20 weeks. The mechanism, causes, and geographic prevalence of this transition are unknown, and can make managing honey bees in certain regions challenging. We hypothesized that the transition to overwintering is regulated by the forager pheromone, ethyl oleate, when forager bees are relegated to the hive for longer periods of time during poor weather conditions. We exposed bees of different ages and tasks to ethyl oleate in cage conditions and measured accepted markers of overwintering: hypopharyngeal gland size and protein content, fat body weight, longevity, pollen consumption, and *vitellogenin* gene expression. We also investigated a possible mechanism for the increased longevity seen in diutinus bees, by looking at gene expression of an immune gene, *defensin.* We found ethyl oleate only had a significant effect on fat body mass, and the ratio of fat body mass to hypopharyngeal gland protein synthesis in nurse bees. This indicates that ethyl oleate may affect the efficiency of metabolism of consumed protein into fat body stores, and an increased metabolic shift from hypopharyngeal gland protein synthesis to fat body production, allowing young bees to prepare for suboptimal conditions. While these findings indicate that ethyl oleate is likely not the sole cause of a transition to a diutinus state, it is possible that when concomitant with other factors such as gradual decline in brood pheromone, pollen dearth, cold temperatures, and photoperiod, ethyl oleate may contribute to the transition to overwintering, a notion worth investigating further.

Introduction

Honey bees *(Apis mellifera)* are a fascinating example of the extremes of animal behavioral plasticity. One remarkable instance when honey bees display exceptional ability to change their physiology and behavior in response to environmental cues, is during their transition to a diutinus, or long-lived state. These "overwintering" bees can live up to four times longer than the summer cohorts (Fukuda & Sekiguchi 1966). This occurs in honey bees that exist in temperate regions that experience harsh winters, but a similar state has been observed in other subspecies of honey bees in tropical places, during unfavorable periods other than winter (Winston 1980, Seeley & Visscher 1985, Mattila et. al. 2001).

The agriculture industry heavily relies on commercial beekeeping pollination services to provide the world food supply. In the winter of 2019-2020, the beekeeping industry in the U.S. experienced 22.2% colony loss (Bruckner et. al. 2020). Winter may exacerbate the plethora of factors contributing to colony failure including parasites, diseases, poor nutrition, pesticides, and environmental changes (Nguyen et. al. 2011, van Dooremalen et. al. 2012, Le Conte & Najavas 2008, Doeke et. al. 2015). Investigating the dynamics of the transition from short-lived to diutinus bees could aid in management adjustments, possibly leading to improved colony survivorship. Studying the physiology and behavior of winter bees, and the environmental factors that influence the transition to an overwintering state, will also help elucidate the geographic prevalence and origin of "overwintering." In addition to potential apicultural applications, understanding regulators of behavioral plasticity in a social insect could greatly advance the fields of gerontology, immunology, animal behavior, ecology, and evolutionary biology.

Honey bee colonies are made up of approximately 40,000 sterile female workers, a single reproductive queen, and male drones (Seeley 1989). In summer, worker bees undergo stereotypical

age-related task transitioning known as temporal or age polyethism. Until 10-15 days after emergence, bees do in-hive tasks like rearing brood and cleaning cells, after which point, they begin performing out-of-hive tasks like guarding, and foraging for food and other resources (Seeley & Kolmes 1991, Huang et. al. 1994). During the summer, there is continual population turnover as old bees die at approximately six weeks of age (Fukuda & Sekiguchi 1966) and are replaced by new bees. In late summer and early fall, brood rearing by the workers and egg laying by the queen slows until there is no brood in the hive (Allen & Jeffree 1956, Jeffree 1956, Shehata et. al 1981). When the temperature drops below -10°C, all the bees cluster within the hive and vibrate to thermoregulate (Stabentheiner et. al. 2003). Unlike shorter-lived summer bees, winter bees can live up to 20 weeks (Fukuda & Sekiguchi 1966) and are behaviorally and physiologically distinct (Fluri et. al. 1982).

In addition to clustering behavior and cessation of brood rearing, there are clear physiological differences in these different states. In summer, young bees have low levels of juvenile hormone (JH), and as they age, these levels rise; foragers have the highest levels of JH (Robinson 1987, Robinson & Ratnieks 1987). Young nurse bees in summer have large hypopharyngeal glands (HPGs) and high vitellogenin (Vg) hemolymph levels (Fluri et. al. 1982). HPGs are glands in the heads of bees that produce proteinaceous secretions (reviewed by Standifier 1967). These secretions are fed to the brood, the queen, workers, and drones, in various concentrations mixed with other foods like pollen or honey (Hrassnigg & Crailsheim 1998, Crailsheim 1991). Consumption of pollen allows the HPGs to develop and enlarge (Standifier 1967, Brouwers 1983, Hrassnigg & Crailsheim 1998) but only exposure to brood pheromone (BP) causes the HPGs to activate and begin producing protein (Huang & Otis 1989, Huang et. al. 1989, Mohammedi et. al. 1996, Peters et. al. 2010, Sagili & Pankiw 2009, Pankiw et. al. 2008).

In contrast, winter bees have large HPGs, yet the glands are inactive and do not synthesize protein (Brouwers 1983, Fluri et. al. 1982), possibly due to the lack of brood (and consequently BP) in the hive during winter. Winter bees also have low juvenile hormone, which is associated with decreased metabolism (Huang et. al. 1994, Huang et. al. 1995), and high vitellogenin hemolymph levels, similar to young bees in summer (Fluri et al. 1982, Doeke et. al. 2015). Both factors may make it easier for winter bees to store fat to overwinter more successfully (Smedal et. al. 2009, Amdam & Omholt 2002). Hemolymph Vg levels are also one of the best predictors of longevity (Kunc et. al. 2019), as some researchers suggest that Vg may recruit hemocytes, improving immunity (Amdam et. al. 2005). Decreased metabolism and increased immunity may partially explain the increased longevity of winter bees.

Winter bees are essentially preserved in a young state for an extended period. It is unknown exactly which bees overwinter, or how this dramatic transition occurs. The queen begins slowing down her laying at some point, but it is unclear if existing bees revert to an overwintering state, or if callow bees emerge pre-determined as winter bees. Furthermore, the literature on this subject suggests there is a cascade of interactions between a multitude of factors that contribute to the transition of this unique physiological and behavioral state including pollen shortage, absence of brood, extended inability to leave the hive due to cold or rain, pheromonally induced changes in demographics, and dropping ambient temperatures (Winston 1980, Mattila et. al. 2001, Mattila & Otis 2007, Huang & Robinson 1995). These questions make functional studies about where and when overwintering occurs difficult to execute.

Several studies have suggested the possibility of worker-worker inhibitory effect caused by adult bees spending an unusually large amount of time in the hive in autumn (Huang & Robinson 1995, Mattila et. al. 2001, Doeke et. al. 2015). They propose that adult bees might

produce a pheromone that contributes to the demographic transition to a winter state in periods of cold weather, rain, reduced daylight, and lack of forage. Most studies conducted on winter bees have been performed in temperate regions with "true winters," consisting of very cold temperatures, reduced daylight, reduced forage, and sometimes heavy precipitation. However, there is evidence of a form of quiescence in tropical regions (Winston 1980, Schmickl & Crailsheim 2001, Maurizio & Hodges 1950, Mattila & Otis 2007). It is unclear how bees behave in intermediary climates such as that of Northern California, for example. The existence of a bee-modulated change in demography, physiology, and task division could also explain how subspecies of honey bees in warmer areas may reap the possible immunological and metabolic benefits of a diutinus state in periods of resource dearth or of heavy rainfall. One possible agent that could lead to the reversion of foragers in fall and the behavioral arrest of nurses is ethyl oleate, which is known to delay the onset of foraging by nurses (Leoncini et. al. 2004).

Ethyl oleate is a non-volatile chemical (Muenz et. al. 2012) produced when foragers consume ethanol from fermented nectar in flowers (Castillo & Maissonase 2012). Ethanol is esterified with oleic acid by $\propto/\beta$ hydrolases. Genes encoding these enzymes are upregulated in foragers compared to nurses, which means foragers have a greater capacity for producing ethyl oleate (Castillo & Chen 2012). Furthermore, JH, which is high in foragers, facilitates the transport of ethyl oleate from the honey crop to the abdominal exoskeleton where it is exuded (Castillo & Maissonase 2012) and perceived by the antennae of nearby bees (Muenz et. al. 2012). If foragers are confined to the hive in inclement weather, there may be high concentrations or increased exposure to ethyl oleate by foragers and nurses alike.

There is evidence that foragers can revert to nursing when needed. Bees that have been foraging for less time are more likely to revert than more experienced foragers (Robinson et. al.

1992, Page et. al. 1992, Amdam & Omholt 2002). That is, that physiological and behavioral plasticity is greater the less time bees have been foraging. When bees revert behaviorally, their JH levels drop and their HPGs develop so they can nurse brood. In the absence of brood, HPGs do not activate, and any consumed protein can go towards building up lipid reserves in fat bodies (Smedal et. al. 2009, Amdam & Omholt 2002). Ethyl oleate is a component of brood pheromone, and when bees are exposed to ethyl oleate, their HPGs have higher protein synthesis (Mohammedi et. al. 1996). Yet, without brood in autumn, increased consumption of pollen protein and higher concentration of ethyl oleate could cause the characteristic developed, yet inactive glands of winter bees. It is also not known whether ethyl oleate can affect foragers and cause behavioral reversion to a winter state. Even without continuous supply of food (i.e., more ethanol to produce ethyl oleate), forager bees are able to inhibit foraging behavior in young bees with artificially high levels of JH (Huang & Robinson 1992), which indicates that perhaps they are able to induce physiological and behavioral changes in fellow foragers. Ethyl oleate modulated hive demography may control polyethism and maintain appropriate division of labor for changing resource availability and brood load in autumn (Leoncini et. al. 2004). This could also have evolutionary connotations relating to the ancestral mechanism of "overwintering" in tropical climates.

It is paramount to survival that the timing of transition to the overwintering state coincides with environmental cues, to ensure appropriate hive demographics. Bees that do not start brood rearing early enough in mid-winter are much slower to pullulate and swarm in the spring of the following year; and late swarms are more likely to starve the following winter (Seeley & Visscher 1985). It is ambiguous if bees in certain areas of even the United States can enter a true overwintering state, since some places, such as central California, experience very mild winters,

yet do have reduced forage. Beekeepers often report seeing a decline in brood rearing, but not necessarily a complete cessation.

Beekeepers and scientists are developing cold storage facilities to reap the purported benefits of overwintering, even in more moderate climates like central California. Properly overwintering may be integral to combating potentially negative consequences of climate change, and brood break can be an effective treatment for the ubiquitous parasitic scourge, *Varroa destructor,* which reproduces in developing brood cells (Ifantidis 1983). Colonies are placed into cold storage in mid-November and removed in early February (DeGrandi-Hoffman et. al. 2019). It has not been experimentally investigated if colonies in cold storage are receiving the proper signals to begin mid-winter brood rearing, so colonies that overwinter in storage facilities may not build up early enough, and they will have reduced winter success the next year (Seeley & Visscher 1985). More research is needed to understand the efficacy of cold storage, the downstream effects on individual and colony health, and if any additional management practices need to be included such as timing, treatment with some pheromone, or pollen supplementation.

Here, we hypothesize that ethyl oleate contributes to the physiological transition of summer bees to diutinus bees (even in tropical areas, or in dearth) including the reversion of already middle-aged or foraging bees to a nurse-like diutinus bee state. We performed two experiments, wherein we exposed bees of different ages and tasks to ethyl oleate in cages and observed the effects on HPG size and protein content, fat body weight, and gene expression of *vitellogenin* and the immune gene, *defensin* (Casteels et. al 1993), all possible or accepted markers of "overwintering" physiology. We also observed worker longevity and consumption of pollen and sugar. This experiment aims to test one possible environmental factor that contributes to the transition to overwintering, and whether this factor is affected by or affects hive demography. Information on

the role that specific factors play, will contribute to further elucidating and completing a model of regulation of transitioning into a diutinus state.

Methods

Bees

All experiments were conducted at the Harry H. Laidlaw Jr. Honey Bee Research Facility at the University of California, Davis. Source colonies were of *Apis mellifera ligustica* genetic background, headed by naturally mated queens (Jackie Park-Burris Queens, Inc.).

Experiment 1 (Figure 1) was conducted in May and June 2020. Frames of emerging brood from a single colony were placed into emergence cages in an incubator at 34.5°C and relative humidity of ~30%. After 24 hours, emerging adults were paint-marked on the thorax with a pen (water-based acrylic paint pen, Uni POSCA). To create five technical replicates, on five consecutive days, 1000 bees per day were paint-marked with different colors. To reduce drift, paint-marked bees were placed into an unrelated, foster colony within a CLAM Corporation 9879 Quick-Set Escape Shelter tent (11.5 ft across and 7.5 ft at center height) (Figure 2) supplied with water and Prosweet (Mann Lake Inc., Woodland, CA) ad libitum (Figure 3). On collection day, 10 randomly selected bees were immediately placed on dry ice as a "baseline" treatment group. At days seven ("nurse" group), 21 (young forager, "YF" group) and 28 (old forager, "OF" group), 200 of the appropriately aged bees were collected from inside the foster colony and placed into Plexiglass cages (14cm x 7cm x 11.5 cm) (Malka et. al. 2007). The first experiment targeted the ages of the bees, rather than the tasks of the bees. These days correspond to the task division, as seven-day old bees should be nurses, 21-day old bees should be in the beginning stages of foraging, and 28-day old bees should have been foraging for a week (Seeley & Kolmes 1991). However, the

bees were not collected while returning from foraging but were rather collected directly from the hive; they were ostensibly not completing these tasks and the division was age based. Therefore, the labels of “Nurse”, “Young Forager” and “Old Forager”, are more appropriately referring to seven, 21, and 28 day old bees. Fifty bees from each age group were assigned to one of the four treatments: ethyl oleate administered in candy (EO Candy), ethyl oleate administered on Grade 1 180 μm thick cellulose filter paper (Whatman) (EO paper), control candy, and control paper. In the first experiment, 98% ethyl oleate (Sigma-Aldrich) was mixed into sugar candy in a concentration of 10mg/gram of candy. 35uL of ethyl oleate was added to the three grams of candy in the EO candy cages each day (Mohammedi et. al. 1996, Leoncini et. al. 2004). 35uL of ethyl oleate was also dropped onto a sheet of filter paper and inserted into the EO paper cages each day. The control paper cages had a dry piece of filter paper added each day, and the control candy had untreated candy added each day. Since the ethyl oleate was 98%, there was no need to add any solvent to the control groups. It was unclear from the literature if bees would be attracted to EO alone, even though antennation is sufficient to perceive it, so we tested if feeding it to them was more effective (Muenz et. al. 2012). In the second experiment in May 2021, no candy treatment was used, so 35uL of ethyl oleate was placed onto a sheet of filter paper and inserted into the treatment cages each day. The control paper cages had a dry piece of filter paper added each day. The bees were fed ad libitum both pollen and candy.

In addition to experimental bees, each cage also contained approximately 100-150 newly emerged bees from 3-4 unrelated colonies to maintain a semi-normal colony level of interaction between bees (Tsvetkov et. al 2019). Each cage was provisioned with three grams of sugar candy (70% powdered sugar and 30% corn syrup), three grams of Bee-Pro Patties+ (Mann Lake), and water ad libitum. To minimize the risk of cross-contamination due to possible volatility of ethyl-

oleate, control and treatment cages were kept in separate VWR incubators set to 34.5 °C and relative humidity of ~30%.

The purpose of the second experiment (Figure 4) was to separate the effects of age and task, and to examine physiological markers reported to be more closely associated with behavior (Huang et. al. 1994). The second experiment was conducted in May 2021. Nurses and foragers from a single source colony were collected and put into cages. Twenty-four hours prior to collection, the colony was turned so that the hive entrance was facing the opposite direction of the neighboring colonies' entrances in order to reduce drift and aggression between focal bees. Bees from each of the age groups were randomly assigned to one of the two treatment groups: ethyl oleate on filter paper (as described above) and control (no ethyl oleate). Since there were no statistically significant differences for any measured parameters when ethyl oleate was applied in candy or on paper, in the second experiment, EO was only administered on paper for simplicity. There were four technical replicates for each age/treatment group, for a total of 16 cages. Cages were provisioned with candy, pollen, water, and callow nurse bees, as described above. Nurse bees were observed on frames putting their heads into open brood cells (Figure 5). Once observed, they were marked with a pen (water-based acrylic paint pen, Uni POSCA) and inserted into a cage. We placed 16 paint marked nurses into each cage. Foragers were collected at midday by placing a barrier over the hive entrance to stop returning foragers from entering. When a forager was observed with either pollen or distended abdomen (indicating nectar collection), it was collected, painted on the thorax, and inserted into a cage. 16 forager bees were placed into each cage. 16 focal bees were placed in each of 16 cages for a total of 256 focal bees. 10 nurse bees and 10 forager bees were immediately placed on dry ice as a "baseline" treatment group.

In the first experiment, each day, the pollen and candy were removed from the cages and weighed and replaced in full. All dead bees were removed, and the number of dead painted bees was recorded. After the bees were in the cages for 7, 10, 14 and 18 days, 10 painted bees were removed from the cage and their heads were immediately removed from their body, placed on dry ice and stored at -80°C for later processing. Occasionally, it was not possible to get all 10 bees, due to high focal bee mortality. One set of cages did not have any bees removed on the sampling days and were simply monitored for longevity. In the second experiment, all surviving focal bees were removed from their cages after 10 days of treatment. Otherwise, processing was identical to the first experiment.

Hypopharyngeal Gland Processing

For dissections, individual heads were transferred directly from dry ice to a dissecting dish and pinned in bee ringer solution (Rangel et. al. 2016). The bee face plate was removed with micro-scissors and forceps and the hypopharyngeal glands were removed in their entirety (Corby-Harris and Snyder 2018). A photo of the glands was taken at 40x magnification with Leica Acquire software at 40x magnification on a Leica M60 dissection microscope. For each bee, Image J was used to calculate the areas of 10 randomly selected, individual acini by outlining the circumference (Figure 6). After dissection, the glands from four bees were pooled, weighed with a Mettler Toledo DeltaRange XPR56 microscale, and transferred to a 1.5mL microcentrifuge tube in 50uL of Tris-buffer (0.1 M Tris–HCl, pH 7.5) for storage at -80°C. To determine the HPG protein content in experiment 1, the Bradford Assay was performed using a Pierce Coomassie Bradford Protein Assay kit by the UC Davis Proteomics Center (Peters et. al. 2010, Mohammedi et. al. 1996, Hartfelder et. al. 2013).

Fat Body Processing

The abdomens of individual bees were placed in preweighed glass test tubes. They were then placed in a Isotemp 60L Oven (Thermo Fisher Scientific) at 70℃ for 48 hours. After 48 hours, the tubes were removed and weighed. Once the dry weight was determined, the abdomens were submerged in pure chloroform for 24 hours and covered with parafilm. After 24 hours, the abdomens were decanted and again submerged in fresh chloroform for another 24 hours. This was repeated for a total of three 24-hour chloroform washes. After the 3rd wash, the final chloroform was decanted, and the abdomens were again placed into the drying oven at 70℃ for another 48 hours. After 48 hours, the tubes were reweighed. The difference in the weight before and after chloroform washes is taken to be the fat body weight (Hopkins 2021).

Gene Expression Analysis

Thoracic tissue was used for analyzing gene expression of *defensin* and *vitellogenin* genes, because vitellogenin hemolymph levels are typically high in winter bees, and *defensin* is associated with immune function. Thoraces were used because the heads were already used for HPG analysis and the abdomens were already used for fat body analysis, so it was the only remaining tissue. Paint marks on thoraces were removed with acetone, wings and legs detached, and thoraces rinsed in bee ringer solution (Rangel et. al. 2016). Thoraces of individual bees were placed in 400uL of Invitrogen TRIzol™ Reagent (ThermoFisher Scientific) in 2.0 mL BeadBug homogenizer tubes with 3.0 mm zirconium beads. They were homogenized in a BeadBlaster™ 24 Microtube Homogenizer (Benchmark Scientific) at 7.00m/s for one minute. After extraction, DNA was removed with a RNA Clean and Concentrator-5 kit (ZYMO Research).

25uL reactions using 1000 ng RNA were used for reverse transcription. Samples were annealed at 70℃ for five minutes, placed on ice, then incubated for 60 minutes at 37℃ (BioRad T100 thermal cycler). cDNA was diluted in water to a final cDNA concentration of 10ng/uL.

qPCR was performed in 2ng/uL 10uL reactions using BioRad SYBR Sso Advanced Green Supermix, using BioRad CFX384. Samples were run in triplicate and included RPL8 and actin (Sigma-Aldrich) as housekeeping genes. Each gene had a no template control and a no reverse transcriptase control, each also run in triplicate. qPCR reactions consisted of a 95℃ (10 minutes) annealing stage and forty cycles of 95 ℃ (15 seconds), 60℃ (30 seconds), and 72℃ (30 seconds), followed by a melt-curve analysis. qPCR primer sequences (Sigma-Aldrich) used were: RPL8.F (5'TGGATGTTCAACAGGGTTCATA3'), RPL8.R((5'CTGGTGGTGGACGTATTGATAA3') (Collins et. al 2004, Evans et. al. 2006), Actin.F(5'TGCCAACACTGTCCTTTCTG3') Actin.R(5'AGAATTGACCCACCAATCCA3') (Chen et. al. 2005), Defensin.F (5' TGTCGGCCTTCTCTTCATGG3'), Defensin.R(5'TGACCTCCAGCTTTACCCAAA3') (Yang et. al. 2005), Vitellogenin.F(5'GTTGGAGAGCAACATGCAGA3'), Vitellogenin.R(5'TCGATCCATTCCTTGATGGT3') (Amdam et. al. 2004).

Statistics

All statistical analyses were performed in MATLAB Version 9.6.0.1072779 (R2019a). In the first experiment, there was no significant difference between the technical cage replicates for any metric, so for all further analyses, they were combined. Likewise, there was no significant difference between control candy and control paper or between EO paper and EO candy, so they were combined for all further analyses into control and EO treatment groups regardless of

application method. All sampling days were combined for all the analyses (except baseline, Day 0 bees, which were excluded from significance testing).

Since it was necessary to pool the HPGs for four bees to determine protein content, the acini area for the same four bees were also pooled in order to compare total protein mass to average acini area (10 acini per bee, measured across both HPGs = 40 total acini for each data point). Even when HPG data was not being compared to protein mass, the data remained pooled by four bees. HPG protein concentration was determined with BCA analysis (UC Davis Proteomics Center), and then converted to mass using the volume of dilution (900uL), in order to compare to fat body mass. Outliers for fat body data were removed based on a 1% threshold on both the upper and lower quantiles (2% removed total). The sum of fat body mass and HPG protein mass was calculated, then the percentage of fat body mass of this total "metabolic output" was found. For comparison to protein mass, the same four bees' fat body mass was also pooled. Total protein mass was used across all analyses of HPG protein, regardless of comparison to fat body mass.

In order to identify the characteristically large (acini area) and empty (protein mass) HPGs of winter bees (Brouwers 1983), we first plotted protein mass against acini area for all ages and sampling days. To find the linear regression of the acini area vs. HPG protein mass, a robust least squares fit was used. Baseline bees were also used in determining the line of fit. We then plotted a line of best fit ($y(x)=mx$) for all ages (including baseline bees), omitting the constant term (y-intercept), in order to account for the biological reality that degenerated, nonexistent acini cannot hold protein (if the acini size were 0, there would be nowhere for the protein to be) (Brouwers 1982). For these data we found the R^2 value for the line of best fit, whereas when each age group was individually compared to this line, and an R^2 value was determined, but it was not a "best fit" to the data, merely a comparison of each age group to the overall dataset line of best fit. We

expected winter bees to have a low protein mass to acini area ratio (close to zero) and appear in the lower right of the graph (x>y). To determine if any bees were in this region, the ratio of protein mass: acini area was found. Bees in this region would have a low ratio.

Fold change was determined from raw amplification data using Real Time PCR Miner Comparative Analysis software (Zhao et. al. 2005), which automatically removes outliers and directly calculates efficiency and CT from raw fluorescence data based on the kinetics of individual PCR reactions without the need for thresholds or a standard curve and uses the first positive second derivative maximum from a logistic model for CT determination (Ruijter et.al. 2013, Zhao et. al. 2005). Since there was no difference in significance when compared to *rpl8* or *actin*, all data presented uses *rpl8* as a reference gene.

For the pollen and candy consumption, time series were smoothed using a 3-day moving median filter for denoising and outlier removal. All comparisons of two groups of data (for example ethyl oleate vs. control for nurses) were done using the nonparametric Wilcoxon-Mann-Whitney test, whereas for more than two groups (for example Ethyl Oleate for Nurse, YF, and OF), the nonparametric Kruskal-Wallis test was used. Neither of these tests assume normality, so we did not check for normality in any of our analyses. The difference in survival probability between treatment groups was performed using the Cox Proportional hazards regression model. For all significance tests, $p < 0.05$ was used as the significance level. All plots display 95% confidence interval error bars.

Results

Experiment 1

Food Consumption

Table 1. Wilcoxon-Mann-Whitney Comparison of Pollen and Candy Consumption by Treatment for Each Age Group (Figures 19-21) (* indicates significant values)

	Nurses	**Young Foragers**	**Old Foragers**
N	16 (8 EO, 8 Control)	15 (8 EO, 7 Control)	15 (8 EO, 7 Control)
Avg. Candy p-value	0.624	0.837	0.0205*
Max. Candy p-value	0.898	0.966	0.324
Avg. Pollen p-value	0.00451*	0.145	0.0429*
Max. Pollen p-value	0.00389*	0.322	0.427

There was a significant difference in average daily and maximum daily consumption of pollen in the nurse age group, and in average daily pollen consumption in the old foragers. Since the food consumption data were of low sample size, significance is not reliable unless confirmed in both parameters (average and maximum) (Faber & Fonseca, 2014).

Table 2. Kruskal-Wallis Comparison of Pollen and Candy Daily (Average and Maximum) Consumption by Age for Each Treatment Group (Figures 22-25) (* indicates significant values)

	Ethyl Oleate	**Control**
N	24	22
Avg. Candy p-value	0.00924*	0.0439*
Max. Candy p-value	0.33	0.83
Avg. Pollen p-value	0.000174*	0.00103*
Max. Pollen p-value	0.0734	0.000435*

Average daily candy and pollen consumption was significant between age groups, but only the maximum daily pollen consumption for the control group was significant.

Hypopharyngeal glands

Average acini area was largest in the baseline bees for each age group and decreased over time while the bees were in cages. The average acini area for baseline bees was largest in nurses, and smallest in old foragers (N = 96: Control Nurse = 16, EO Nurse = 16, baseline Nurse = 2; Control YF =15, EO YF = 16, baseline YF= 2; Control OF = 12, EO OF = 15, baseline OF = 2; Figure 6). Similarly, the total protein mass was highest in the baseline bees and decreased upon caging. The total protein mass for the baseline bees was largest in nurses, and smallest in old foragers as well (N = 96: Control Nurse = 16, EO Nurse = 16, baseline Nurse = 2; Control YF = 15, EO YF = 16, baseline YF = 2; Control OF = 12, EO OF = 15, baseline OF = 2; Figure 7).

The equation for the line of best fit for protein mass vs. average area of 10 acini for all ages is $y = 24.63x$, $R^2 = 0.60$ (N= 96: Control Nurse= 16, EO Nurse = 16, baseline Nurse = 2; Control YF = 15, EO YF = 16, baseline YF = 2; Control OF = 12, EO OF = 15, baseline OF = 2; Figure 8).

Table 3. Wilcoxon-Mann-Whitney Comparison of All Hypopharyngeal Gland Parameters by Treatment for Each Age Group (Figure 13-15) (* indicates significant values)

	Nurses	**Young Foragers**	**Old Foragers**
N	32 (EO 16, Control 16)	31 (EO 16, Control 15)	27 (EO 15, Control 12)
Average Acini Area p-value	0.235	0.0921	0.678
Total HPG Protein Mass p-value	0.068	0.0649	0.542
Protein Mass:	0.023*	0.594	0.272

Average Acini Area p-value			
Residual Protein Mass p-value	0.048*	0.678	0.232

Table 4. Kruskal-Wallis Comparison of All Hypopharyngeal Gland Parameters by Age for Each Treatment Group (Figures 12, 16-18) (* indicates significant values)

	Ethyl Oleate	**Control**
N	47	43
Average Acini Area p-value	0.0125*	0.00464*
Total HPG Protein Mass p-value	0.0101*	2.48×10^{-5}*
Protein Mass: Average Acini Area p-value	0.00192*	9.76×10^{-6}*
Residual Protein Mass p-value	4.56×10^{-6}*	0.00131*

Fat Bodies

Fat body mass did not decrease from baseline as noticeably as acini area or HPG protein mass (Figure 33).

Table 5. Wilcoxon-Mann-Whitney Comparison of Fat Body Mass by Treatment for Each Age Group (Figure 34-36, 38-40) (* indicates significant value)

	Nurses	**Young Foragers**	**Old Foragers**
N	126 (64 EO, 62 Control)	121 (63 EO, 58 Control)	97 (51 EO, 46 Control)
Fat Body Mass p-value	0.041*	0.659	0.01*
N	32 (16 EO, 16 Control)	31 (16 EO, 15 Control)	25 (13 EO, 12 Control)
Fat Body Percentage of Total p-value	0.00499*	0.567	0.289

(HPG Protein + Fat Body)			

Table 6. Kruskal-Wallis Comparison of Fat Body Mass by Age for Each Treatment Group (Figure 37, 42)

	Ethyl Oleate	**Control**
N	178	166
Fat Body Mass p-value	0.0342*	0.0175*
N	45	43
Fat Body Percentage of Total p-value (HPG Protein + Fat Body)	0.0027*	$1.12 \times 10^{--05}$*

Gene Expression

Table 7. Wilcoxon-Mann-Whitney Comparison of Gene Expression by Treatment for Each Age Group (Figures 26-27)

	Nurses	**Young Foragers**	**Old Foragers**
N	120 (EO 61, Control 59)	117 (EO 61, Control 56)	89 (EO 50, Control 39)
***Defensin* p-value**	0.238	0.443	0.565
N	128 (EO 62, Control 66)	118 (EO 61, Control 57)	89 (EO 50, Control 39)
***Vitellogenin* p-value**	0.527	0.1	0.425

Table 8. Kruskal-Wallis Comparison of Gene Expression by Age for Each Treatment Group (Figure 28-29)

	Ethyl Oleate	**Control**
N	172	154
***Defensin* p-value**	$p= 2.21 \times 10^{-11}$*	3.73×10^{-11}*

N	173	156
***Vitellogenin* p-value**	6.26×10^{-14}*	2.86×10^{-12}*

Survival Probability

Table 9. Cox Proportional Hazards Regression Model of Survival Probability for Each Age Group (Figures 30-32)

	Nurses	**Young Foragers**	**Old Foragers**
N	150 (EO 75, Control 75)	168 (EO 76, Control 92)	131 (EO 63, Control 68)
Survival Probability p-value	0.487	0.0183*	0.24

Experiment 2

Table 10. Wilcoxon-Mann-Whitney Comparison of All Data by Treatment for Each Age Group

	Nurses	**Foragers**
N	64 (EO 31, Control 33)	27 (EO 11, Control 16)
Average Acini Area p-value	0.3104	0.4590
N	62 (EO 30, Control 32)	25 (EO 9, Control 16)
Fat Body Mass p-value	0.9831	0.0468*
N	80 (40 EO, 40 Control)	80 (40 EO, 40 Control)
Daily Pollen Consumption p-value	0.5487	0.8534

While the difference was not significant, there was a 9.96% increase in median EO nurse average acini area. Due to high mortality in the EO Forager group, the sample size is incredibly low, so the fat body mass significance value is likely not reliable.

Table 11. Wilcoxon-Mann-Whitney Comparison of All Data by Age for Each Treatment Group

	Ethyl Oleate	**Control**
N	42 (Nurse 31, Forager 11)	49 (Nurse 33, Forager 16)
Average Acini Area p-value	0.0035*	0.1273
N	39 (Nurse 30, Forager 9)	48 (Nurse 32, Forager 16)
Fat Body Mass p-value	0.0043*	0.5694
N	80 (40 Nurse, 40 Forager)	80 (40 Nurse, 40 Forager)
Daily Pollen Consumption p-value	0.0554*	0.0220*

Nurse bees had higher median average acini area and fat body mass values. They also consumed more pollen each day.

Discussion

Under our experimental conditions, there was an indication that ethyl oleate may contribute to the modulation of specific physiological markers of overwintering, as well as the metabolic function which might lead to characteristic increased longevity of overwintering bees. We hypothesized that the transition to an overwintering physiological state was precipitated by foragers spending more time in the hive, due to suboptimal weather conditions such as shorter days, colder temperatures, forage dearth, or rain, leading to build up of in-hive concentration and increased exposure to ethyl oleate; and that ethyl oleate, on its own, in the absence of brood (brood pheromone), could promote a physiological reversion of foragers to a nurse-like or winter bee state and promote the physiological arrest of nurses, allowing protein consumption (dwindling pollen and cannibalization of brood (Schmickl & Crailsheim 2001, Amdam & Omholt 2002)) to build up fat reserves in the fat bodies rather than production of protein in the HPGs for brood feeding (Smedal et. al. 2009, Amdam & Omholt 2002).

In the first experiment, ethyl oleate caused higher overall fat body mass in all age groups (significantly in nurses and old foragers) compared to controls. The nurses treated with ethyl oleate also had a significantly lower protein mass to acini area ratio than controls. The nurses treated with ethyl oleate also had higher fat body percentage of total "metabolic output" mass (HPG protein mass and fat body mass) compared to control nurses, even though they ate significantly less pollen. The percentage of total "metabolic output" mass composed of fat bodies increased in all ages over time from a minimum in the baseline bees. That is, in an absence of brood and with pollen consumption, the metabolism seems to shift from HPG protein synthesis to fat body storage, in all age groups regardless of treatment with EO. However, EO intensified the shift of metabolism of consumed protein from HPG protein synthesis to fat body storage synthesis (significantly in nurses) (Smedal et. al. 2009, Amdam et. al. 2002). Pollen may have been sufficient to maintain large acini area in all age groups regardless of EO treatment (Standifier 1967, Brouwers 1983, Hrassnigg & Crailsheim 1998), but EO seems to allow any consumption of pollen to build up fat reserves more than HPG protein production. EO may encourage what little dwindling protein autumn and dearth bees are able to acquire to build up fat storage more efficiently.

The most biologically relevant characteristic of diutinus bees is longevity, which ethyl oleate did not have a significant effect on. There was no significant difference in survival between treatment groups, except in young foragers, where ethyl oleate exposed bees had slightly lower survival than controls. There was, however, a difference in survival between age groups; young foragers lived the longest (67 days vs. 61 days in both nurses and old foragers). This may have been due to the fact that nurses spent an overall longer period of time in the cages, and that old foragers had lower tolerance or plasticity to adapt to cage stress by the time they were placed in the cages. This indicates that even if ethyl oleate does impact metabolism, to encourage

overwintering traits, it is not the sole driver of the transition, because exposure to ethyl oleate alone did not impact longevity in these conditions.

Ethyl oleate did not have any effect on the amount of candy consumed in any of the age groups. This indicates that the ethyl oleate administered in candy did not have an attractive or deterring effect on the amount of candy consumed. Since the food consumption data were of low sample size, significance was not reliable unless confirmed in both parameters (average and maximum) (Faber & Fonseca, 2014) so, likely the only truly significant difference between ethyl oleate and control was in the nurse pollen consumption, where controls ate more pollen than ethyl oleate treated bees. Each age group had a significant difference in the amount of both protein and candy consumed, which is supported by prior findings that different ages and tasks of honey bees have different metabolic functions (Huang et. al. 1994) and dietary requirements (Winston 1987).

In the first experiment, there was no difference in *vitellogenin* gene expression between treatment groups, but the distributions were different between age groups. Studies of true overwintering bees found higher levels of vitellogenin hemolymph levels, rather than higher gene expression of *vitellogenin,* which are not always correlated (Dainat et. al. 2012, Amdam et. al. 2002). Our data agree with prior findings that differently aged bees have different levels of *vitellogenin* (Fluri et. al. 1982) and ethyl oleate did not affect this trend. The differential expression between age groups was also seen for *defensin.* It is well documented that gene expression of *defensin* varies between age groups (Lin et. al. 2022), and our results are no different: treatment with ethyl oleate had no impact on this trend, and there was no significant effect of ethyl oleate within age groups.

Across all age groups, there was a positive correlation between protein concentration and acini size, indicating that large acini had more protein content and activity. No individuals, in either

control or treatment groups, displayed the overwinter traits of large acini and low glandular protein content (Fluri et. al. 1982). In our experimental environment, the lack of brood would presumably cause a decline in protein production over time, as brood pheromone from the hive began to wear off (e.g., Brouwers 1983). Since there was no brood or brood pheromone present in the incubators, it is expected that protein production would remain low, and decrease over time, which was observed. We did see a steady decline in both size of acini and protein concentration in all age groups and treatment groups over time. There is no indication that ethyl oleate prolonged enlargement of the acini.

In the first experiment, it was unclear if there was a true physiological distinction between the bees of different ages (Nurse, Young Forager, Old Forager) because the "foragers" were not observed actually foraging. Despite this, the results across all metrics indicate that there were physiological differences between the age groups regardless of lack of active foraging in the tent environment. This indicates that despite no observed behavioral differences in task performance in the hive, age was sufficient to impact physiology.

In the second experiment, we isolated the effect of task rather than age to determine if there would be a difference in the effect of ethyl oleate or more noticeable physiological differences between age groups than seen in the first experiment. While not significant, the nurses tended to have larger fat bodies and average acini areas than foragers, with no significant effect from treatment with EO. We did not measure HPG protein in the second experiment, so it was not possible to analyze the metabolic shift from HPG activity to fat body storage, which was the most significant result in the first experiment. There was high mortality in the EO forager group, forcing the experiment to end prematurely and resulting in low sample size for that group. It is also possible that sampling the bees at 10 days was not enough time for extreme differences in treatment groups

to arise (compared to the 14 and 18 days included in the analysis of the first experiment's data). The results from this experiment are inconclusive and warrant further investigation to disentangle the role of age versus task in establishing overwintering populations.

Our hypothesis that young foragers would demonstrate greater plasticity and show a stronger reaction to EO than the other age groups, was not supported (Amdam et. al. 2002, Robinson et. al. 1992). Rather, these results indicate that if EO does contribute at all to a shift toward overwintering, it may only act on nurse bees. This provides some insight into the demography of which bees overwinter. It seems likely, considering these results, that nurse bees have a great capacity for overwintering, but it remains unclear if older bees are also able to revert to a nurse-like overwintering state. In further studies, it would be necessary to investigate other physiological markers such as vitellogenin hemolymph levels, juvenile hormone, and expression of metabolic genes to understand the mechanism more. It is possible that the sudden shift to experimental conditions does not properly simulate the gradual change of the season that prefaces a transition to a diutinus state (Mattila & Otis 2007). In further studies, it will be necessary to simulate gradual changes like these and investigate the interaction of ethyl oleate with other possible contributing factors such as brood pheromone and pollen load.

Acknowledgements

I want to thank all those that helped me throughout this long, arduous journey, both directly and indirectly with the research as well as emotional and mental support. My greatest appreciation extends to Elina Nino, Alessandra Beelen, Daniel Korenblum, Gabriela Grigorean, Justin Hocke, Lauren Rusert, Charley Nye, Aleen Vartivarian, Jess Woods, Gehrig Loughran, and Ada Sedova.

Figures:

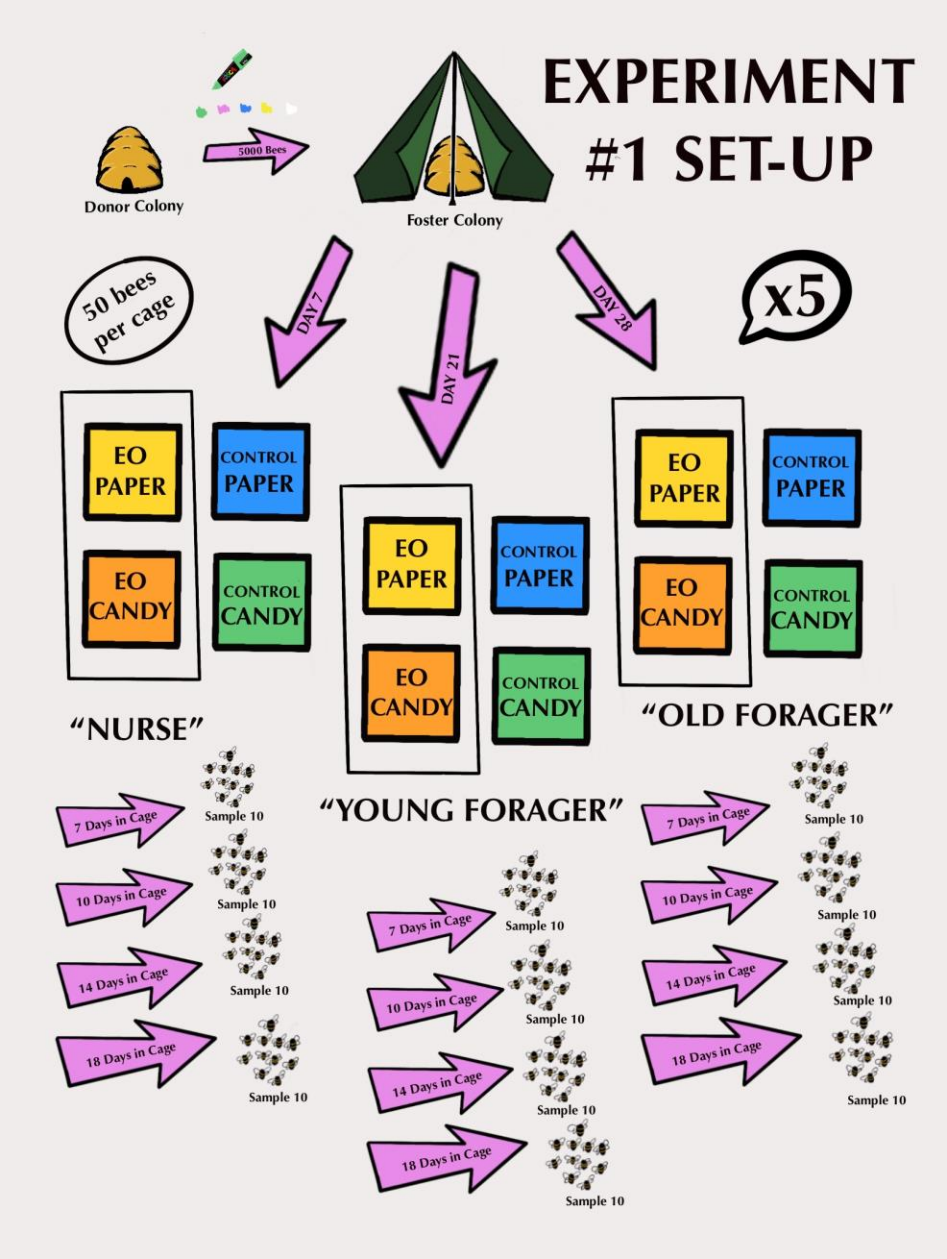

Figure 1. Experiment 1 setup. Newly emerged bees from a donor colony were painted on the thorax then inserted into a transplant colony in a tent. After 7 days, 50 bees were collected and put into cages with newly emerged attendant bees, and separated by treatment (ethyl oleate in candy, ethyl oleate on filter paper, control candy, and control paper). This was repeated after 21 days and 28 days. There were 4 technical replicates, and one set of cages that were not sampled and merely monitored for survival. Once the bees were in their respective cages for seven, 10, 14, and 18 days, up to 10 bees (depending on survival) were removed and placed on dry ice for further processing.

Figure 2. (above) CLAM tent (Amazon.com) used to enclose the foster colony in experiment 1. (below) Foster colony on a hive stand and sugar syrup (Prosweet) feeder inside CLAM tent used in experiment 1.

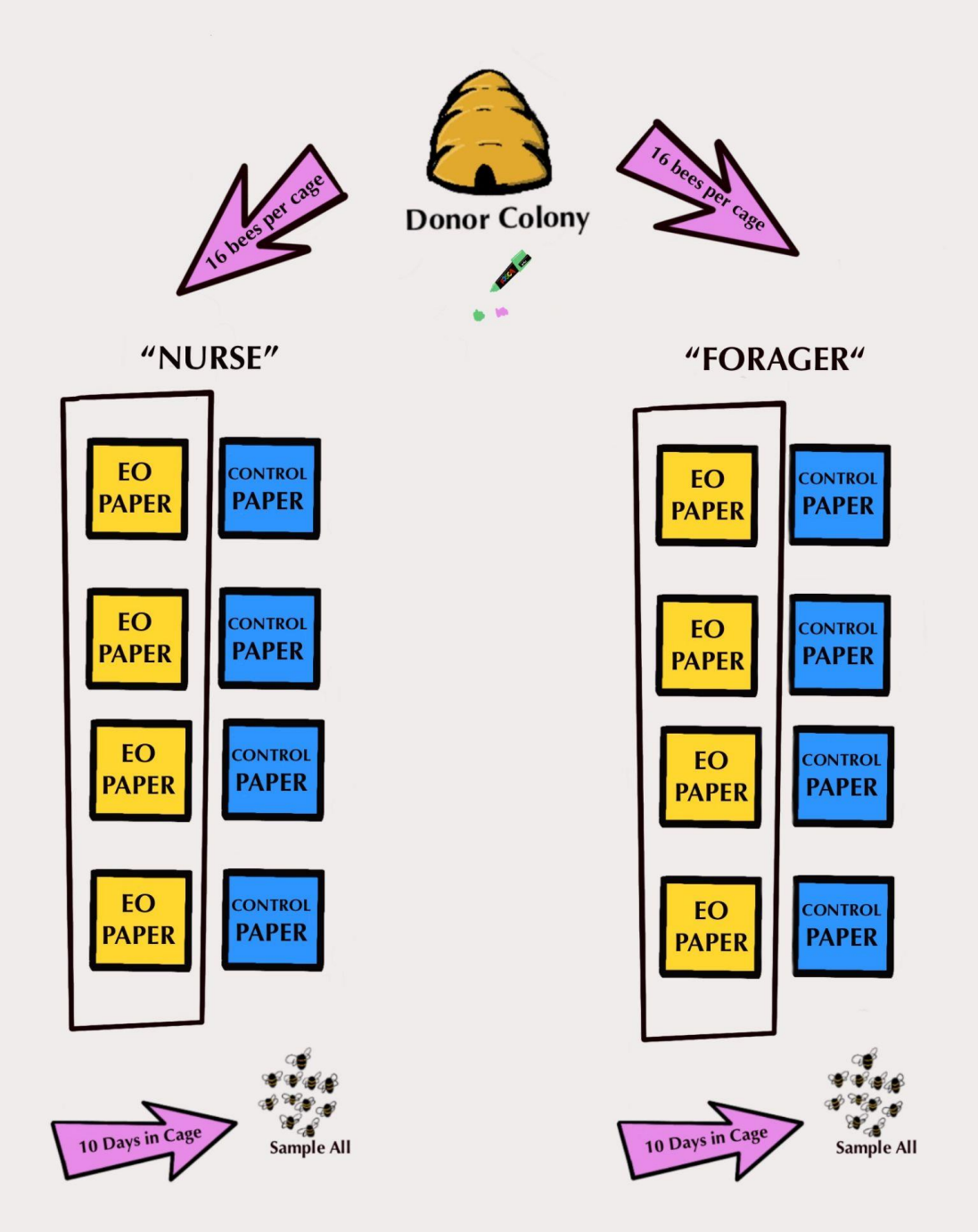

Figure 3. Experiment 2 setup. Nurse bees observed feeding brood were removed directly from frames and forager bees with distended abdomens or pollen loads were collected upon returning to a single donor hive. These were then separated into ethyl oleate and control treatments (in separate incubators) by task. 16 focal (either nurses or foragers) were added to each cage along with newly emerged attendant bees. All focal bees were then collected on dry ice after 10 days in the cage.

Figure 4. Nurse bees insert their heads into open brood cells to feed the larvae. This behavior was observed before selecting bees to paint and insert into cages for experiment 2.

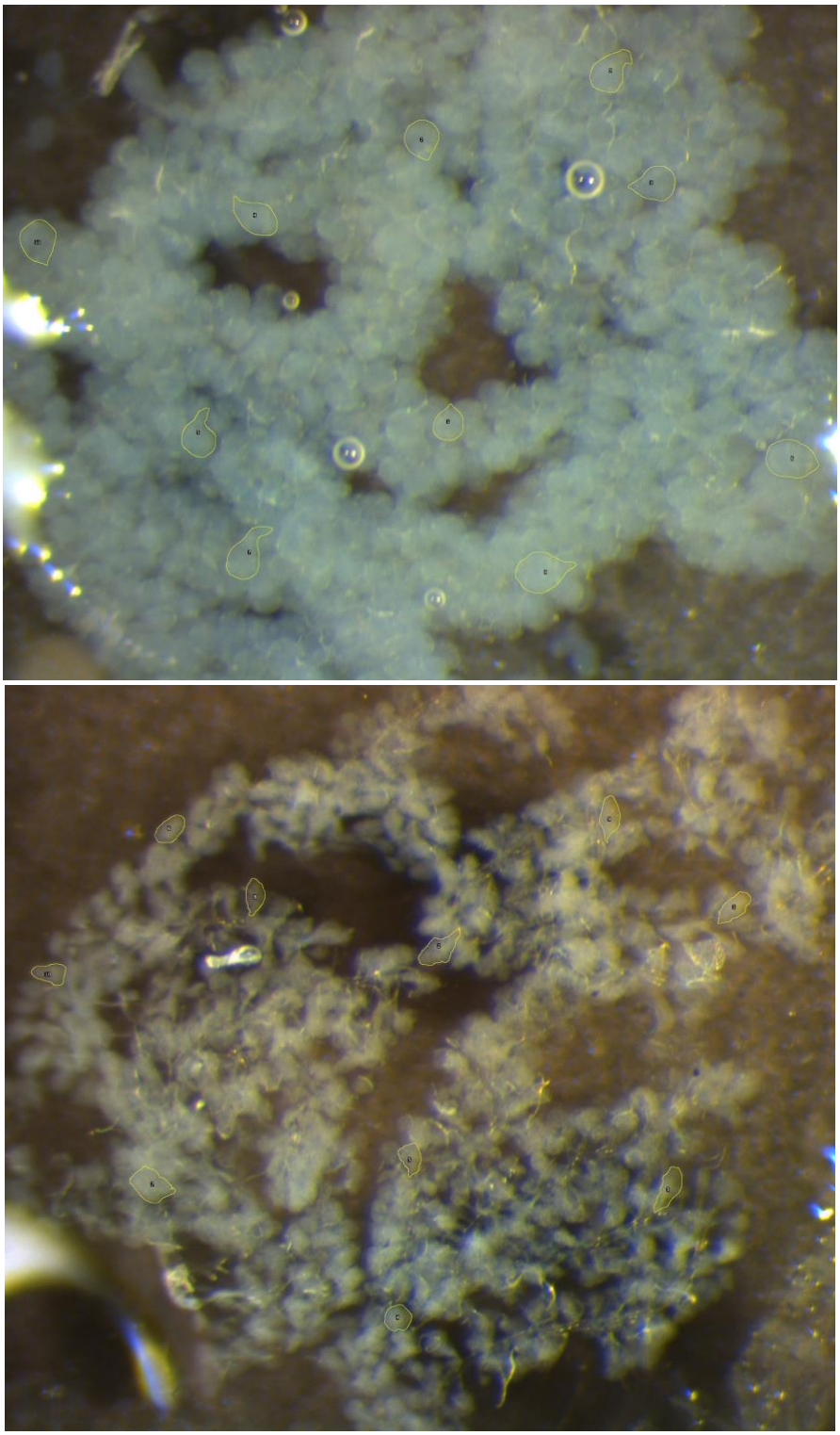

Figure 5. (above) Bee#68, Pink Nurse baseline, with Image J outline showing which acini were measured. Represents large, developed acini. (below) Bee#13 Green Old Forager Day 18 with Image J outline showing which acini were measured. Represents small acini. Both images were captured at 40x magnification, with HPGs in bee ringer solution.

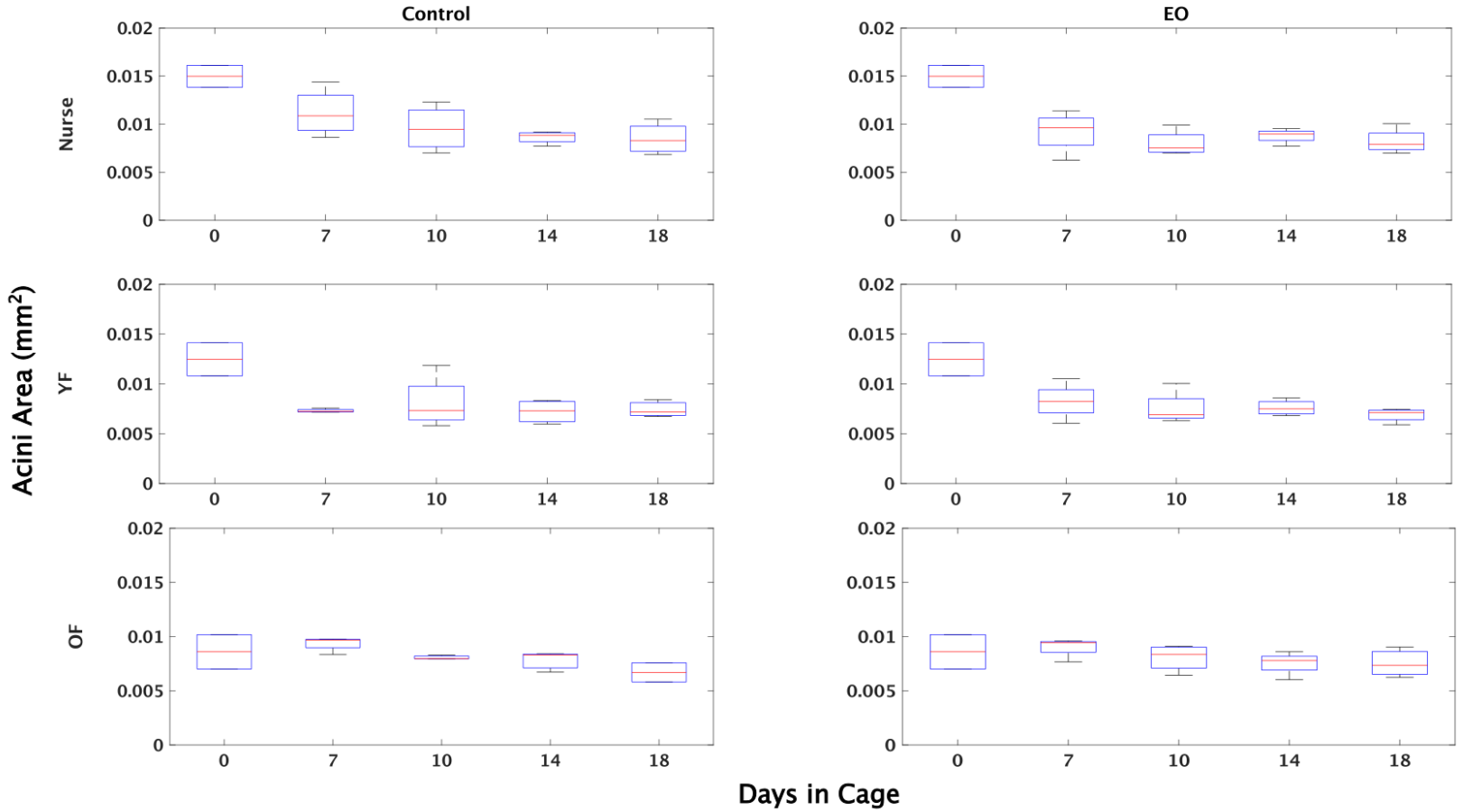

Figure 6. Boxplots of average acini area on each sampling day (each data point represents 4 pooled bees, 10 acini per bee, measured across both HPGs = 40 total acini for each data point). The "baseline" bees are represented by "Day 0" and the baseline bees are the same for both Ethyl Oleate (EO) treatment and Control. N = 96: Control Nurse = 16, EO Nurse = 16, baseline Nurse = 2; Control Young Forager (YF) =15, EO YF = 16, baseline YF= 2; Control Old Forager (OF) = 12, EO OF = 15, baseline OF = 2).

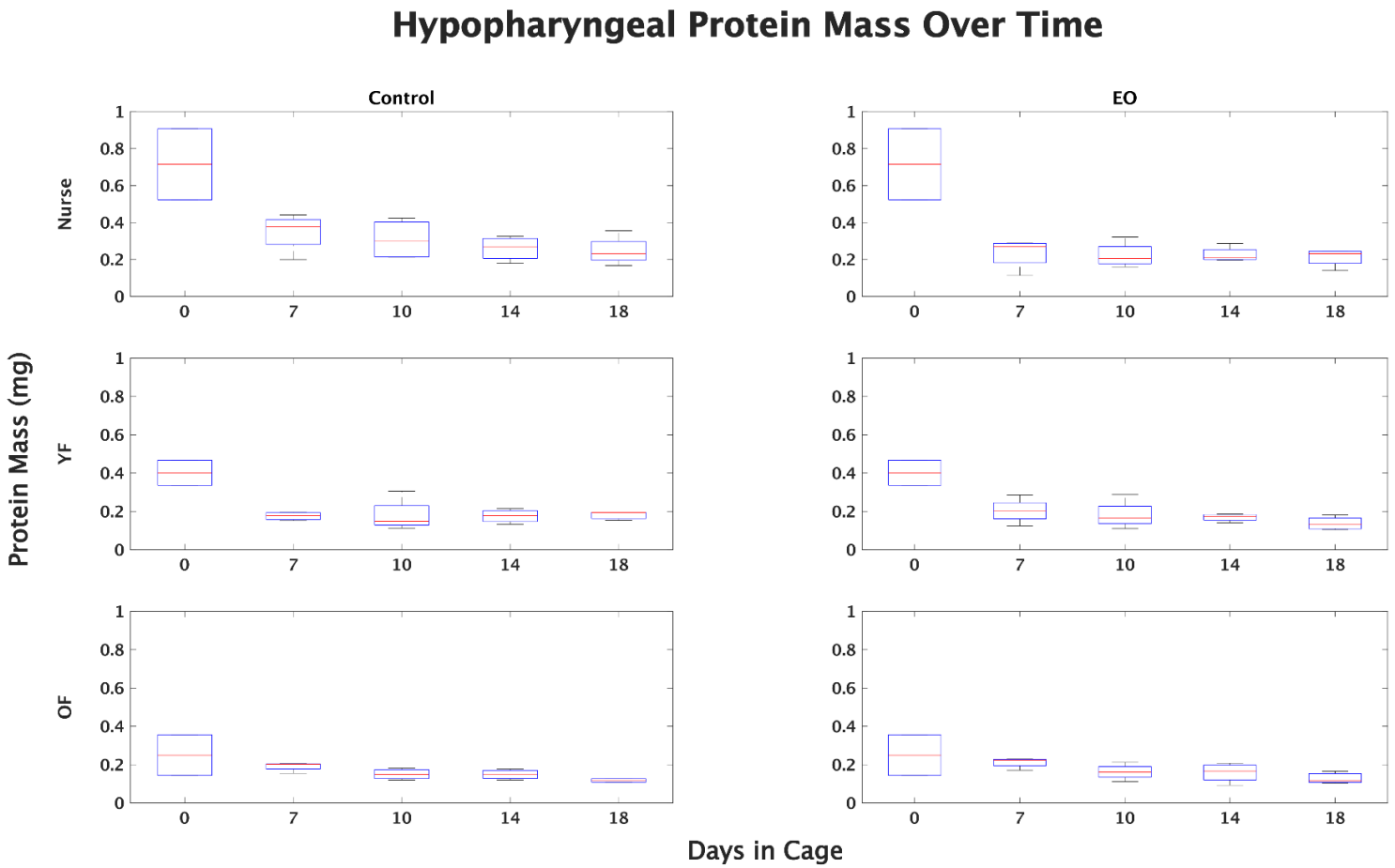

Figure 7. Boxplots of protein mass on each sampling day (each data point represents four pooled bees, 2 HPGs for each bee = 8 HPGs per data point). The baseline bees are represented by "Day 0" and the baseline bees are the same for both Ethyl Oleate (EO) treatment and Control. N = 96: Control Nurse = 16, EO Nurse = 16, baseline Nurse = 2; Control Young Forager (YF) = 15, EO YF = 16, baseline YF = 2; Control Old Forager (OF) = 12, EO OF = 15, baseline OF = 2.

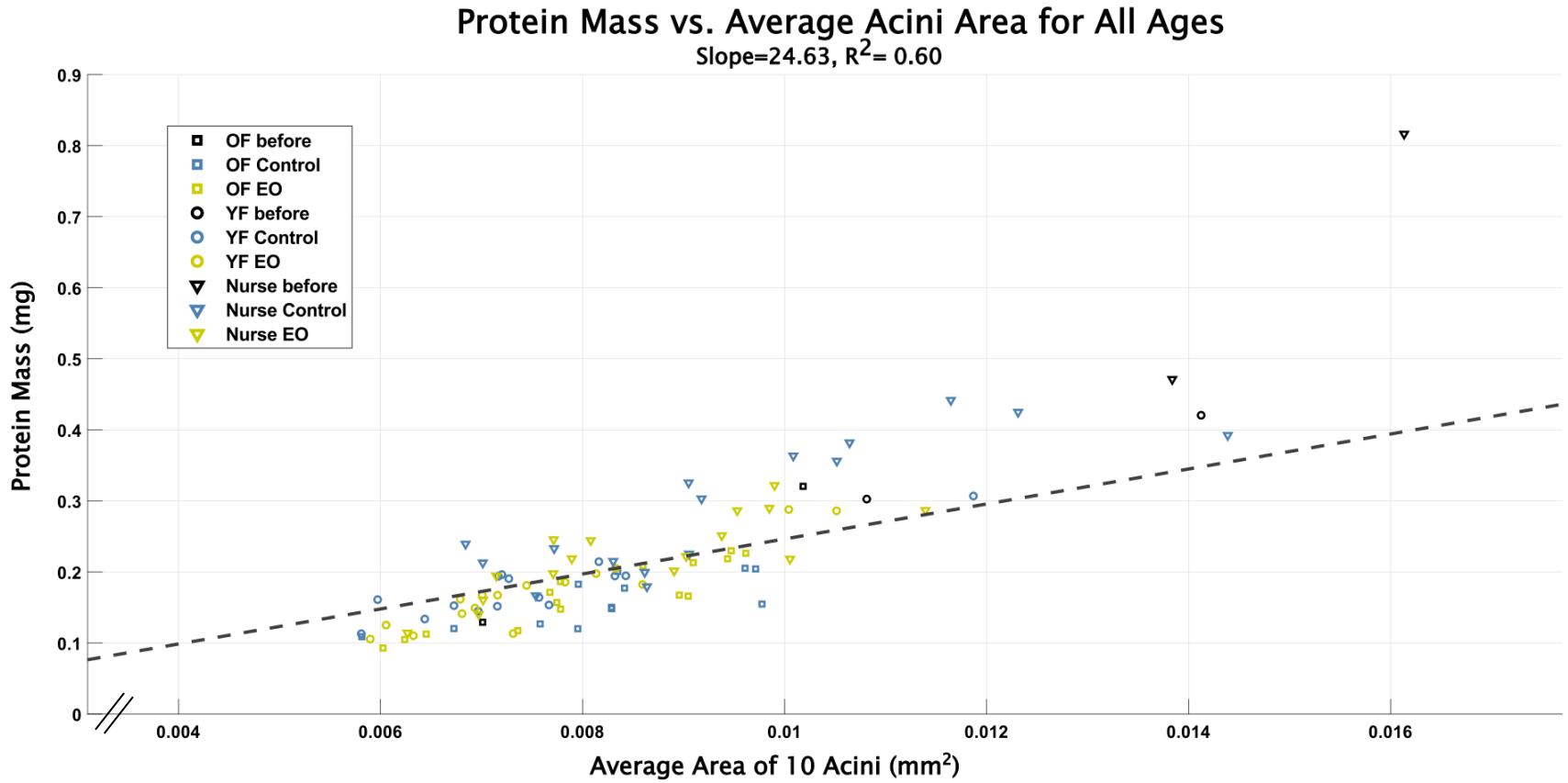

Figure 8. Protein mass vs. average area of 10 acini for all ages. The equation for the line of best fit is y = 24.63x. The y-intercept was adjusted to be zero because an acini of area zero cannot hold any protein. R^2 = 0.60. Each data point represents four bees, because the HPGs of four bees were combined to determine protein mass. N= 96: Control Nurse= 16, Ethyl Oleate (EO) Nurse = 16, baseline Nurse = 2; Control Young Forager (YF) = 15, EO YF = 16, baseline YF = 2; Control Old Forager (OF) = 12, EO OF = 15, baseline (before) OF = 2.

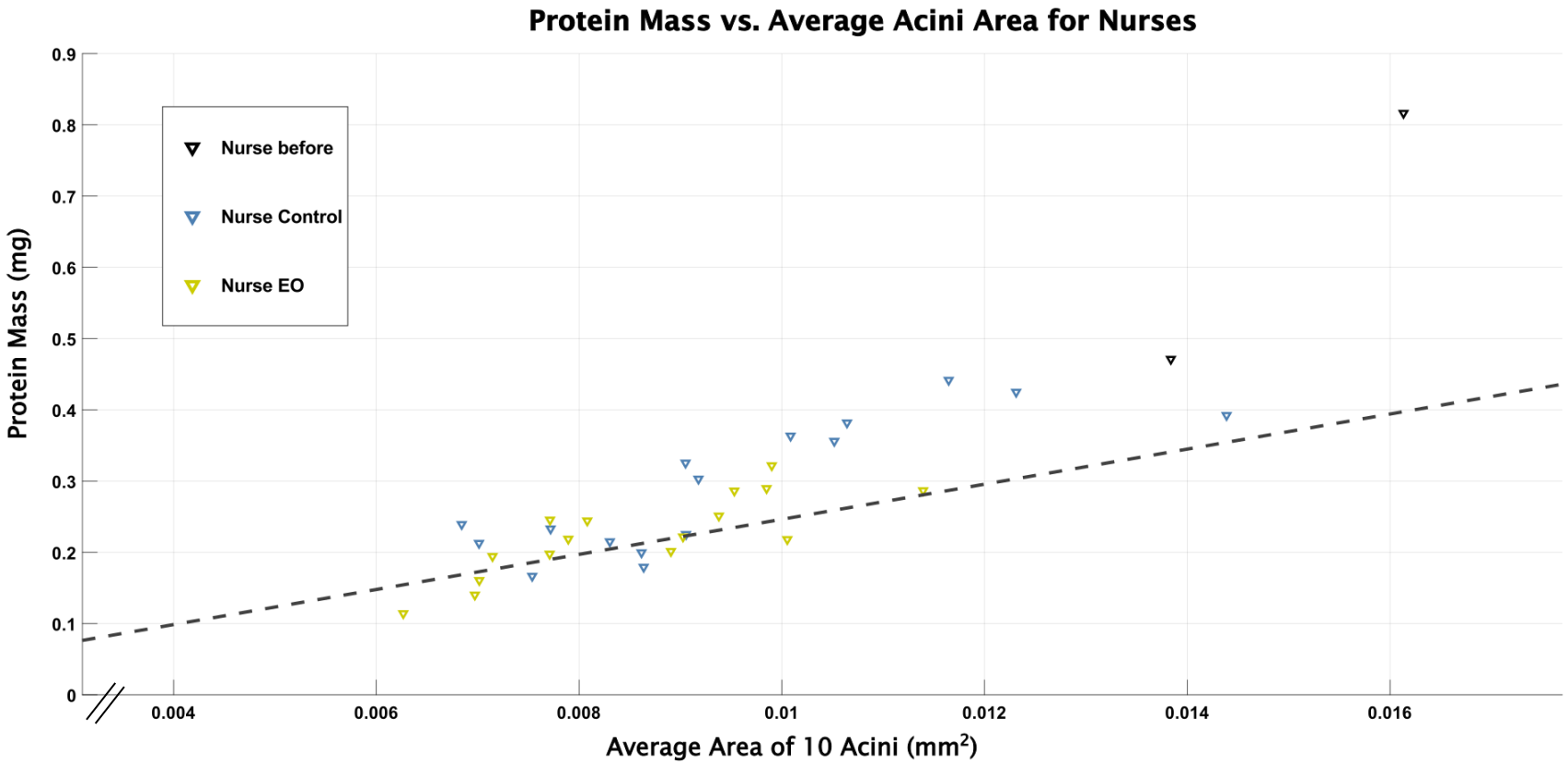

Figure 9. Protein mass vs. average area of 10 acini for nurses. For the equation for the line of best fit for all age groups, $y = 24.63x$, $R^2 = 0.42$. The y-intercept was adjusted to be zero because an acini of area zero cannot hold any protein. Each data point represents four bees, because the HPGs of four bees were combined to determine protein mass. N = 34: Control Nurse = 16, Ethyl Oleate (EO) Nurse = 16, baseline (before) Nurse = 2.

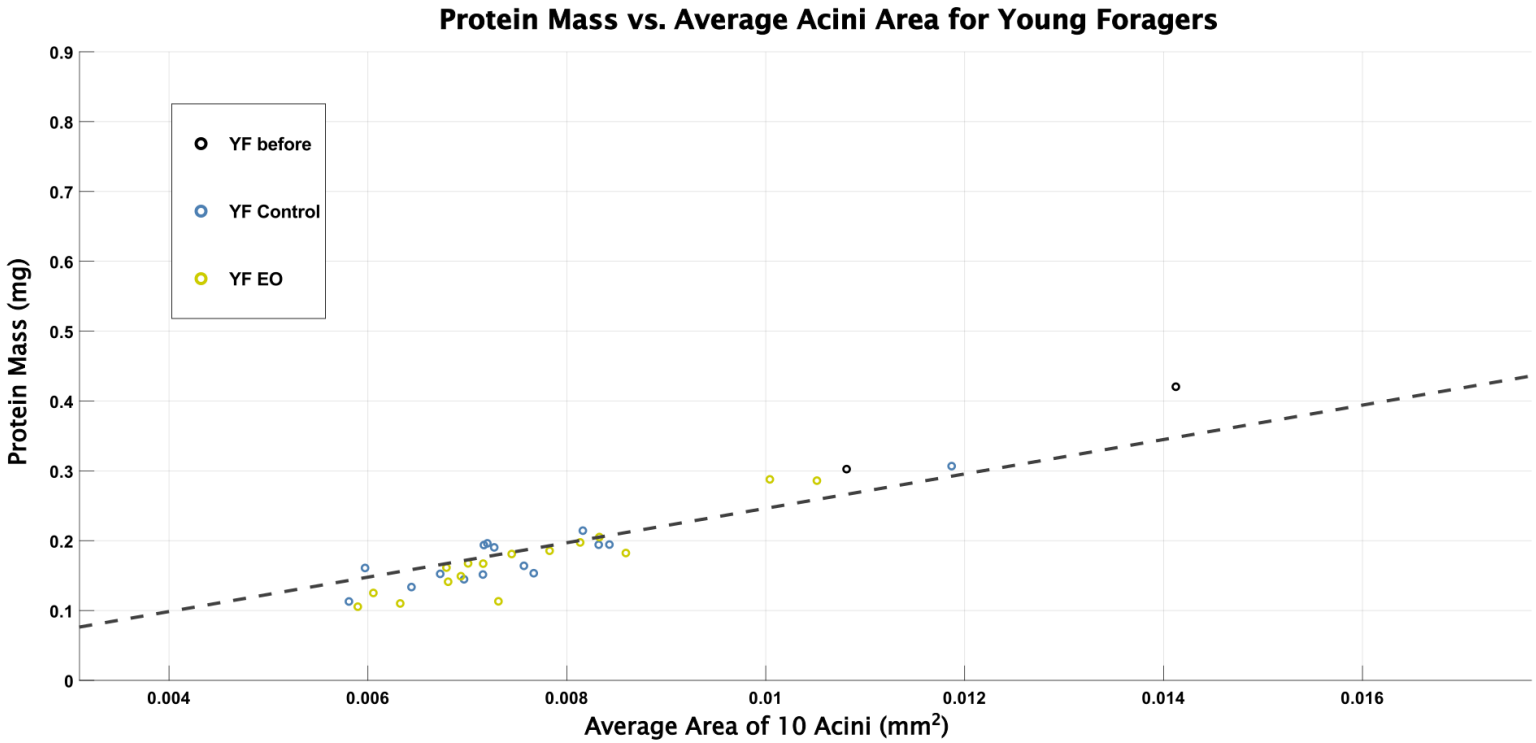

Figure 10. Protein mass vs. average area of 10 acini for Young Foragers (YF). For the equation for the line of best fit for all age groups, $y = 24.63x$, $R^2 = 0.82$. The y-intercept was adjusted to be zero because an acini of area zero cannot hold any protein. Each data point represents four bees, because the HPGs of four bees were combined to determine protein mass. N = 33: Control YF = 15, Ethyl Oleate (EO) YF = 16, baseline (before) YF = 2.

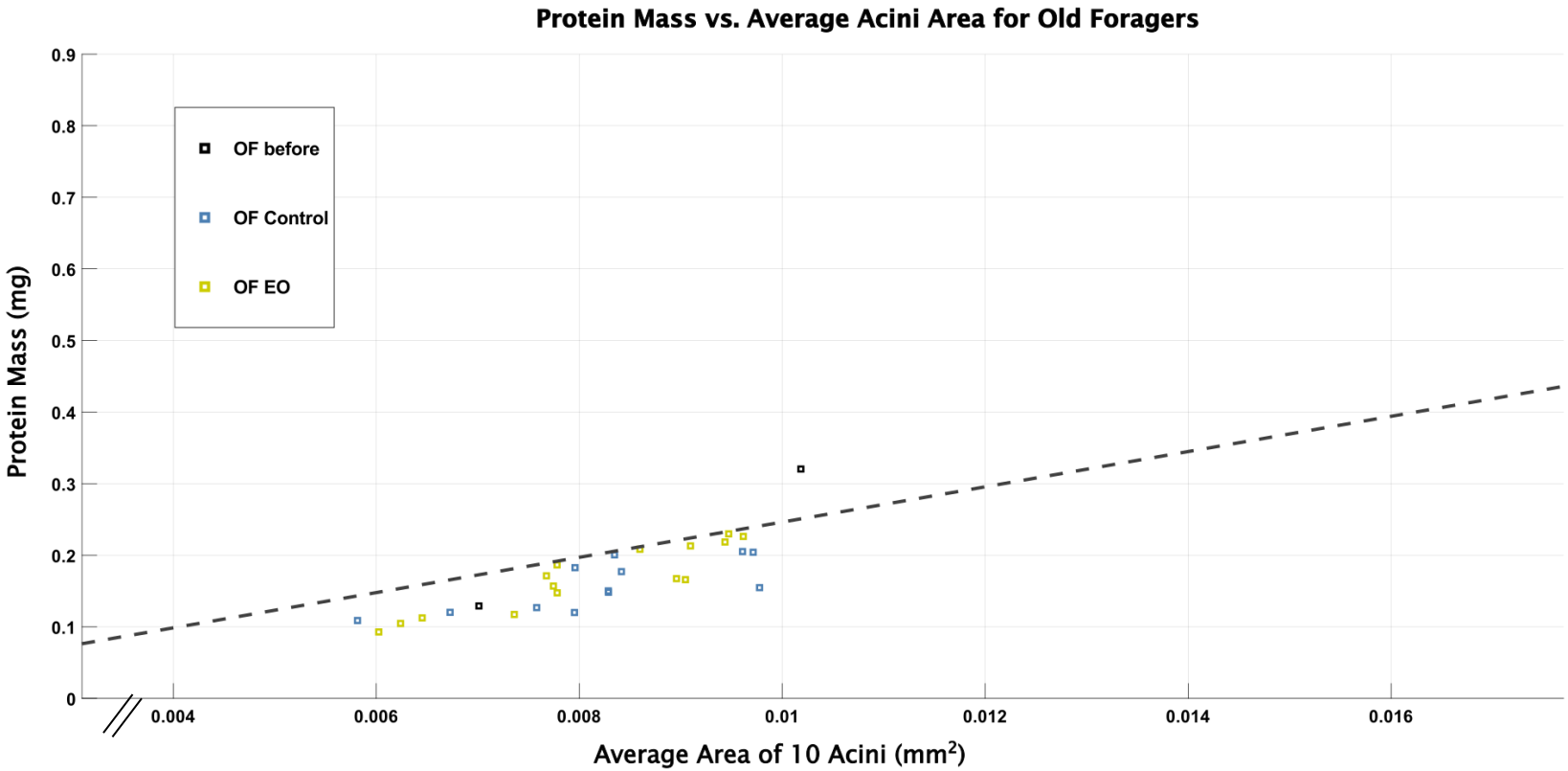

Figure 11. Protein mass vs. average area of 10 acini for Old Foragers (OF). For the equation for the line of best fit for all age groups, $y = 24.63x$, $R^2 = 0.16$. The y-intercept was adjusted to be zero because an acini of area zero cannot hold any protein. Each data point represents four bees, because the HPGs of four bees were combined to determine protein mass. N = 29: Control OF = 12, Ethyl Oleate (EO) OF = 15, baseline (before) OF = 2.

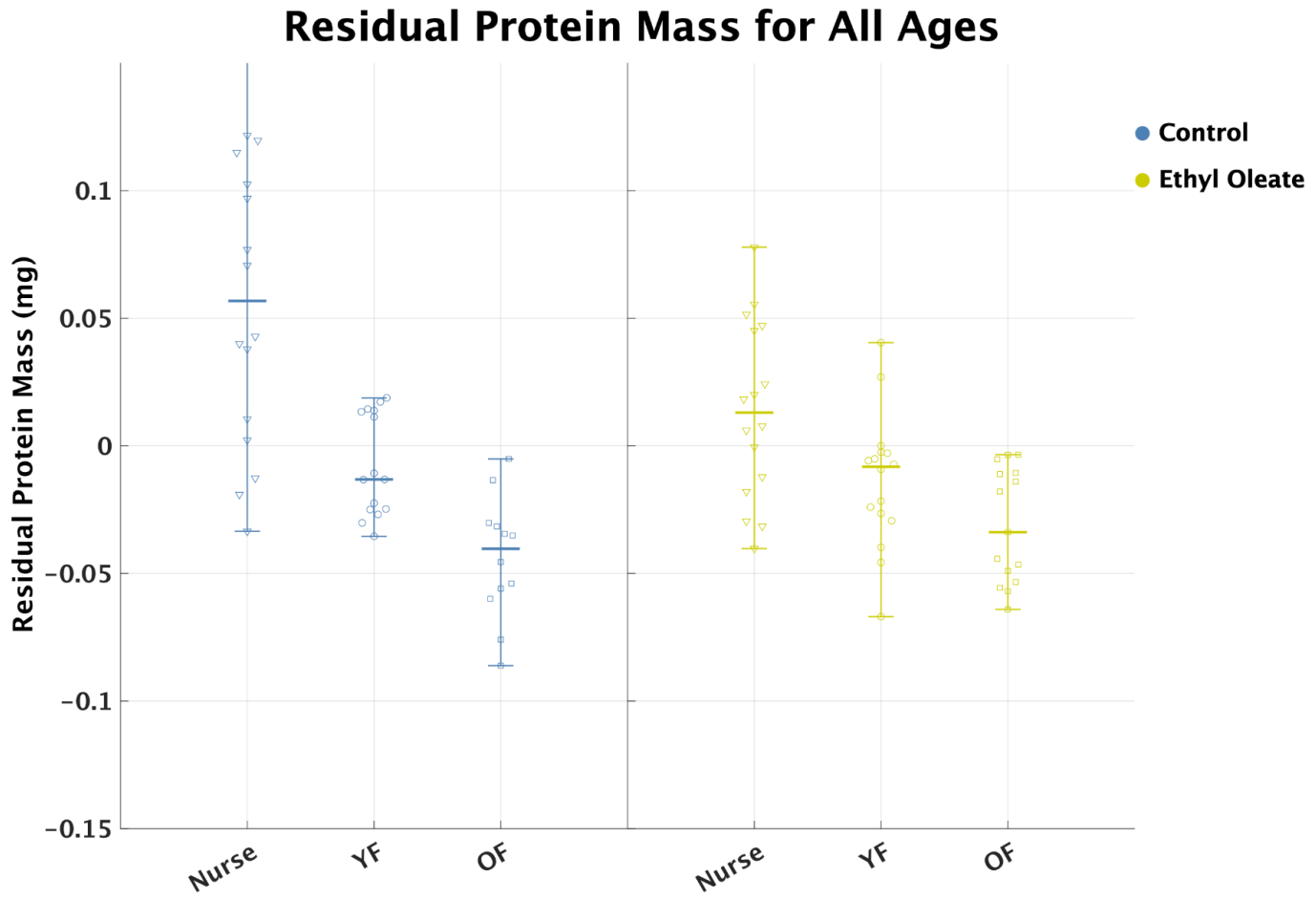

Figure 12. Residuals of the protein mass compared to line of best fit (y = 24.63x). Control p = 4.56 x10^{-6}, Ethyl Oleate (EO) p = 0.00131. Control Nurse median = -0.0568, control Young Forager (YF) median = -0.0132, control Old Forager (OF) median = -0.0403. EO Nurse median = 0.013, EO YF median = -0.082, EO OF median = -0.0338. N = 90: Control Nurse = 16, EO Nurse = 16; Control YF = 15, EO YF= 16; Control OF = 12, EO OF = 15.

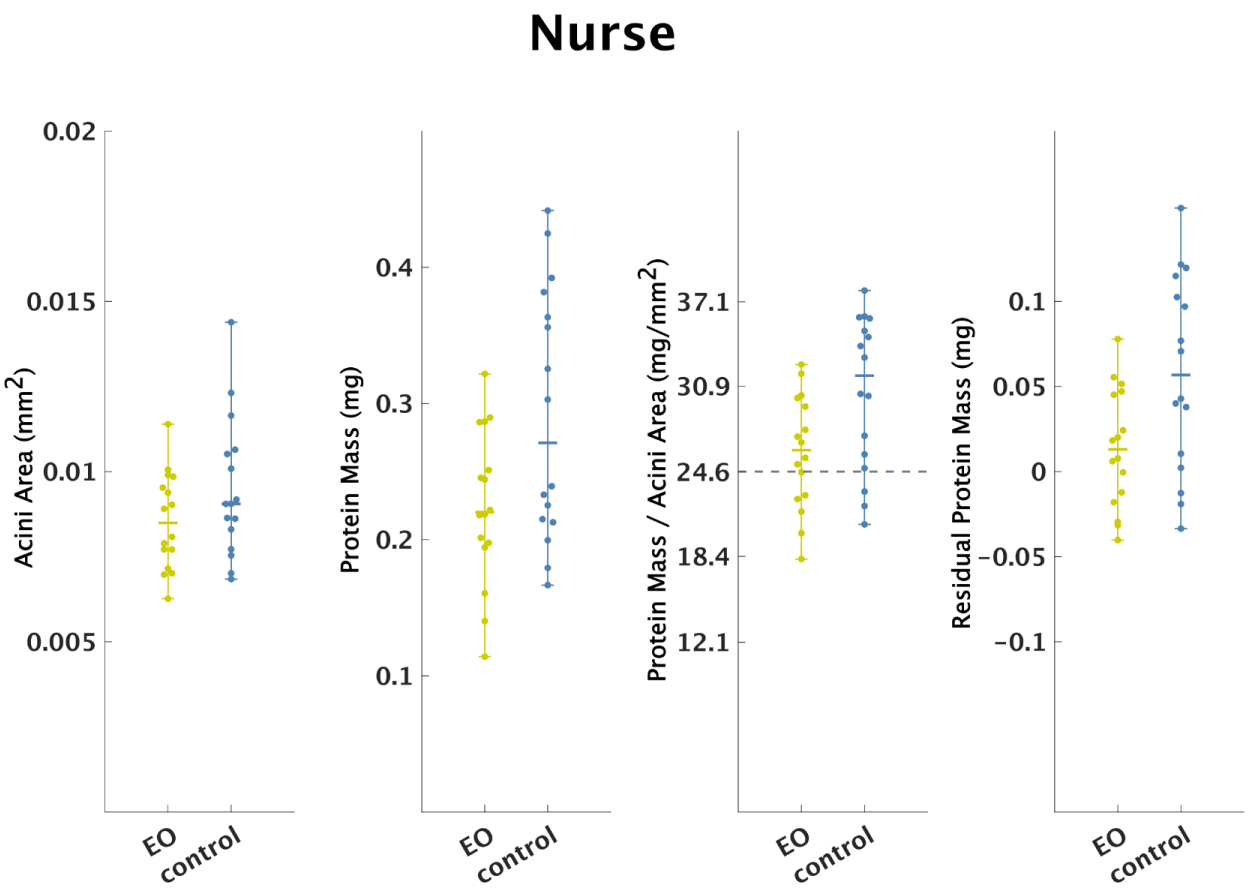

Figure 13. Summary of protein mass and acini area data for nurses. Average acini area for 40 acini (10 from each of 4 bees) p = 0.235; Ethyl Oleate (EO) median = 0.00849 mm^2, control median = 0.00905 mm^2. Total protein mass of 8 HPGs (4 bees) p = 0.068, EO median = 0.22 mg, control median = 0.271 mg. Ratio of total protein mass to average acini area p = 0.023, EO median = 26.2 mg/mm^2 control median = 31.7 mg/mm^2, the dashed line represents the slope of the line of best fit, 24.63 (Figures 8-11). Residual protein mass (to line of best fit) p = 0.048, EO median = 0.013, control median = 0.0568. N = 32: Control Nurse = 16, EO Nurse =16.

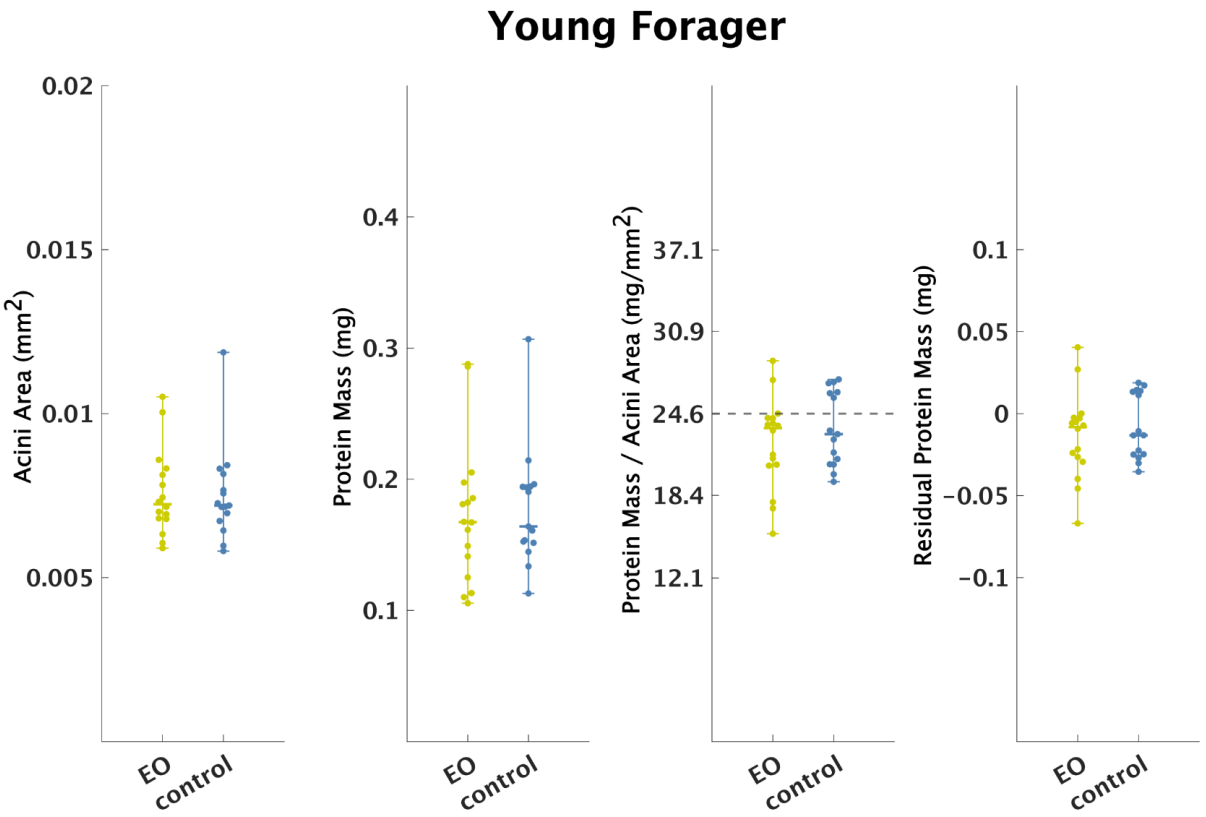

Figure 14. Summary of protein mass and acini area data for Young Foragers (YF). Average acini area for 40 acini (10 from each of 4 bees) p = 0.921 Ethyl Oleate (EO) median = 0.00724 mm^2, control median = 0.0072 mm^2. Total protein mass of 8 HPGs (4 bees) p = 0.649, EO median = 0.167 mg, control median = 0.164 mg. Ratio of total protein mass to average acini area p = 0.594, EO median = 23.5 mg/mm^2 control median = 23. 1 mg/mm^2, the dashed line represents the slope of the line of best fit, 24.63 (Figures 8-11). Residual protein mass (to line of best fit) p = 0.678, EO median = -0.0082, control median = -0.0132. N = 31: Control YF = 15, EO YF = 16.

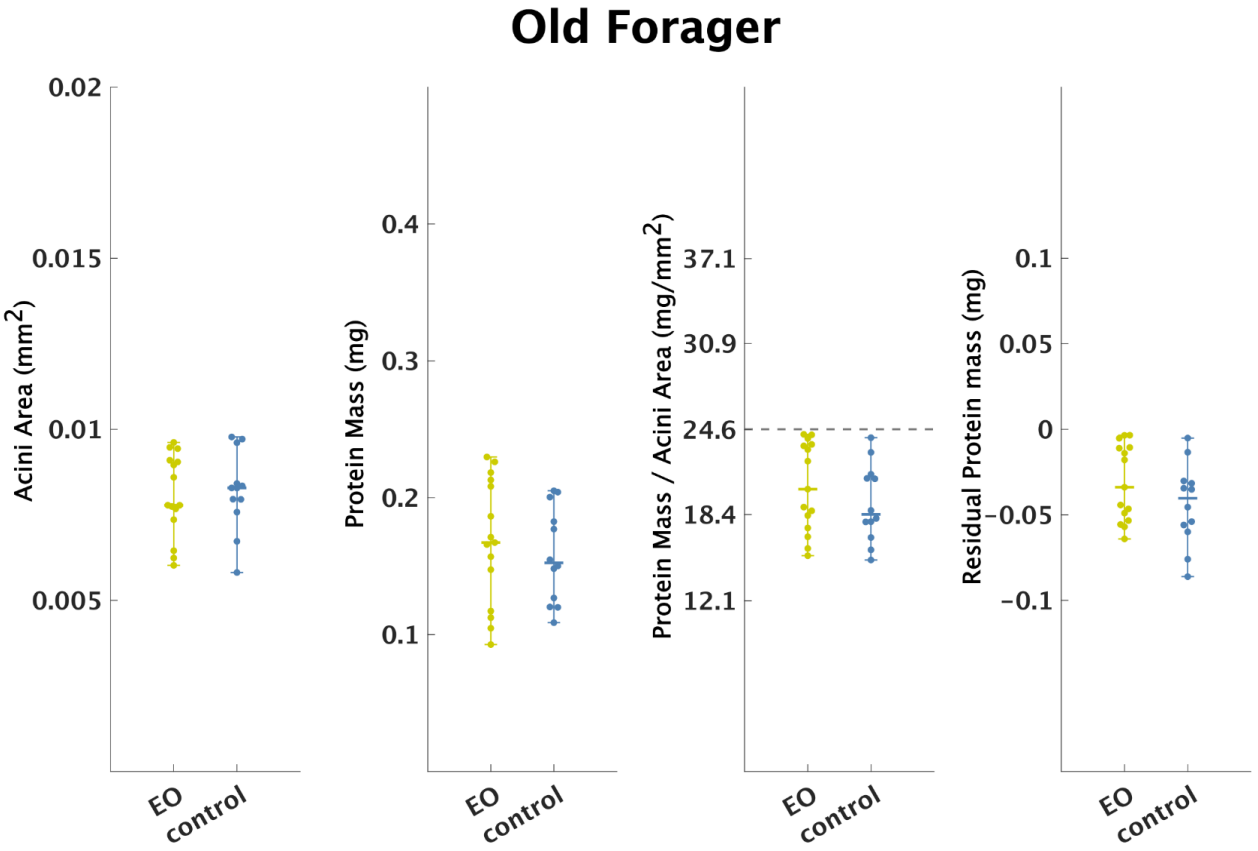

Figure 15. Summary of protein mass and acini area data for Old Foragers (OF). Average acini area for 40 acini (10 from each of 4 bees) p = 0.678 Ethyl Oleate (EO) median = 0.00778 mm^2, control median = 0.00829 mm^2. Total protein mass of 8 HPGs (4 bees) p = 0.542, EO median = 0.167mg, control median = 0.152mg. Ratio of total protein mass to average acini area p = 0.272, EO median = 20.3 mg/mm^2 control median = 18.4 mg/mm^2, the dashed line represents the slope of the line of best fit, 24.63 (Figures 8-11). Residual protein mass (to line of best fit) p = 0.232, EO median = -0.0338, control median = -0.0403. N=27: Control OF = 12, EO OF = 15.

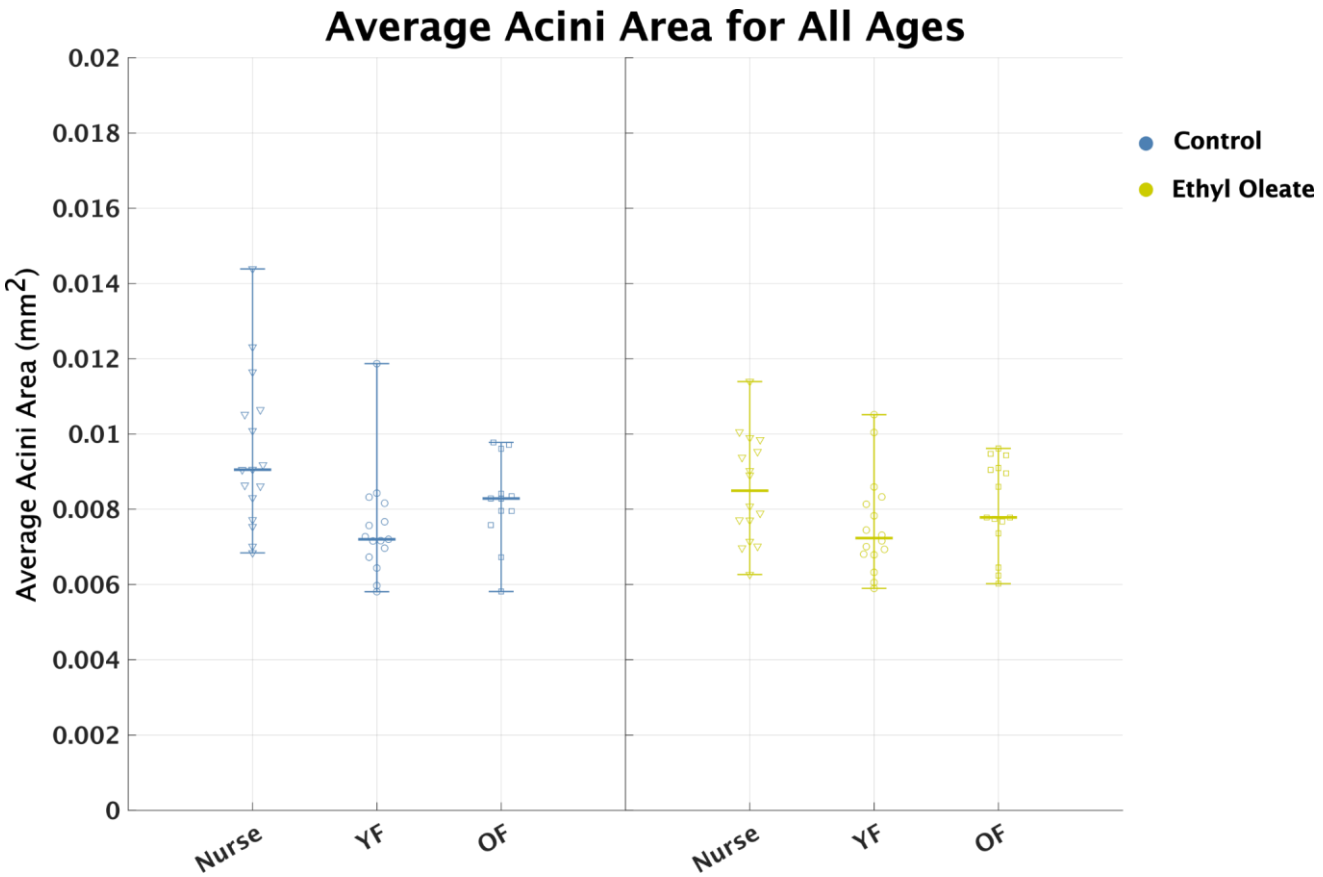

Figure 16. Average area of 40 acini (10 each for 4 bees) for each treatment group, separated by age. A three-way Kruskal-Wallis test yielded p = 0.00464 between age groups for the control treatment, and p = 0.0125 between age groups for the ethyl oleate treatment. Control Nurse median = 0.00905 mm^2, control Young Forager (YF) median = 0.0072 mm^2, control Old Forager (OF) median = 0.00829 mm^2, Ethyl Oleate (EO) Nurse median = 0.00849 mm^2, EO YF median = 0.00724 mm^2, EO OF median = 0.00778 mm^2. N = 90: Control Nurse = 16, EO Nurse = 16; Control YF = 15, EO YF = 16; Control OF = 12, EO OF = 15.

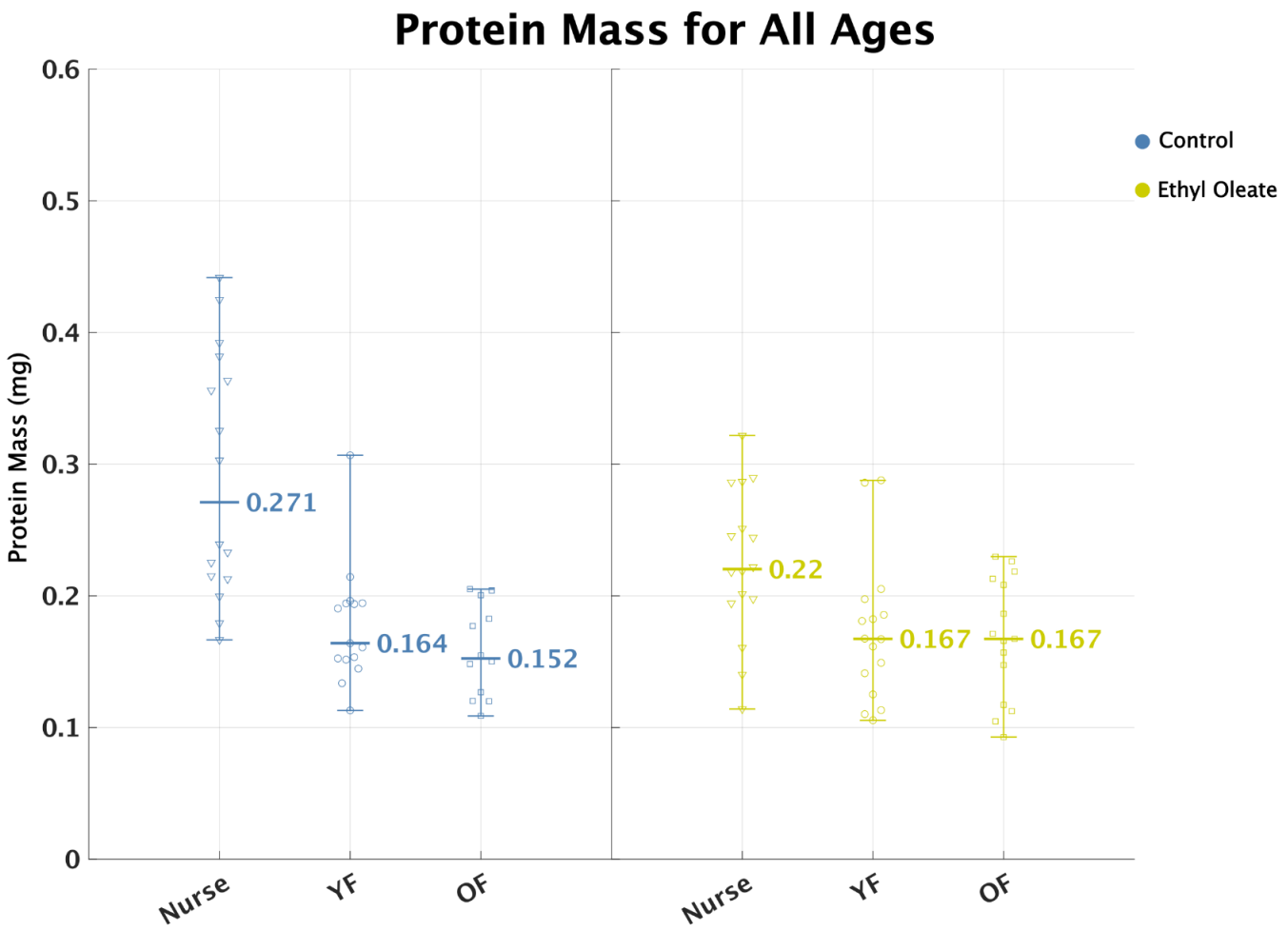

Figure 17. Total protein mass for 8 HPGs (4 bees), separated by age, with median values displayed. A three-way Krsukal-Wallis test yielded $p = 2.48 \times 10^{-5}$ between age groups for the control treatment, and $p = 0.0101$ between age groups for the Ethyl Oleate (EO) treatment. N=90: Control Nurse = 16, EO Nurse = 16; Control Young Forager (YF) = 15, EO YF=16; Control Old Forager (OF) = 12, EO OF = 15).

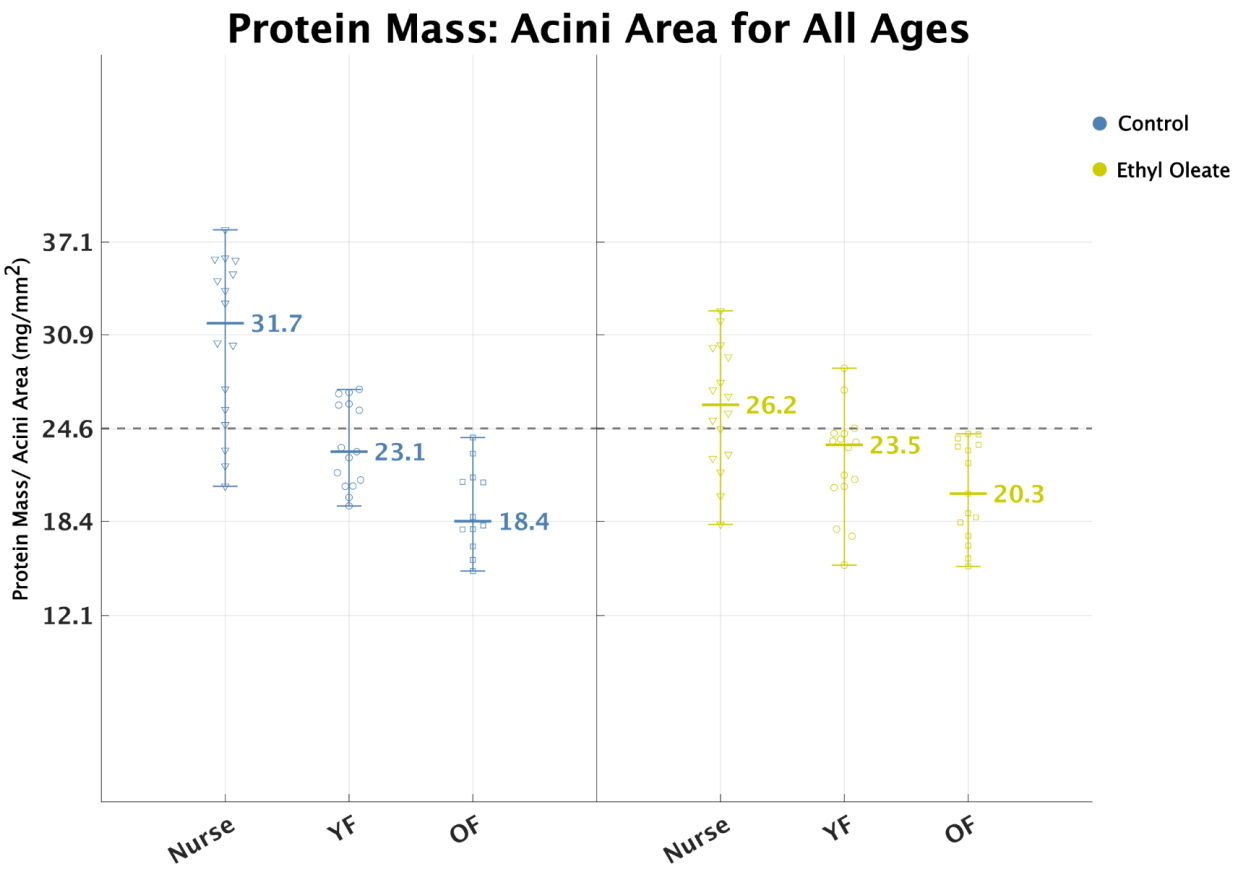

Figure 18. Ratio of protein mass to average area of 10 acini for each treatment group, separated by age, with median values displayed. A three-way Krsukal-Wallis test yielded $p = 9.76 \times 10^{-6}$ between age groups for the control treatment, and $p = 0.00192$ between age groups for the Ethyl Oleate (EO) treatment. The line represents the slope of the line of best fit, 24.63 (Figures 9-12). N = 90: Control Nurse = 16, EO Nurse = 16; Control Young Forager (YF) = 15, EO YF = 16; Control Old Forager (OF) = 12, EO OF = 15.

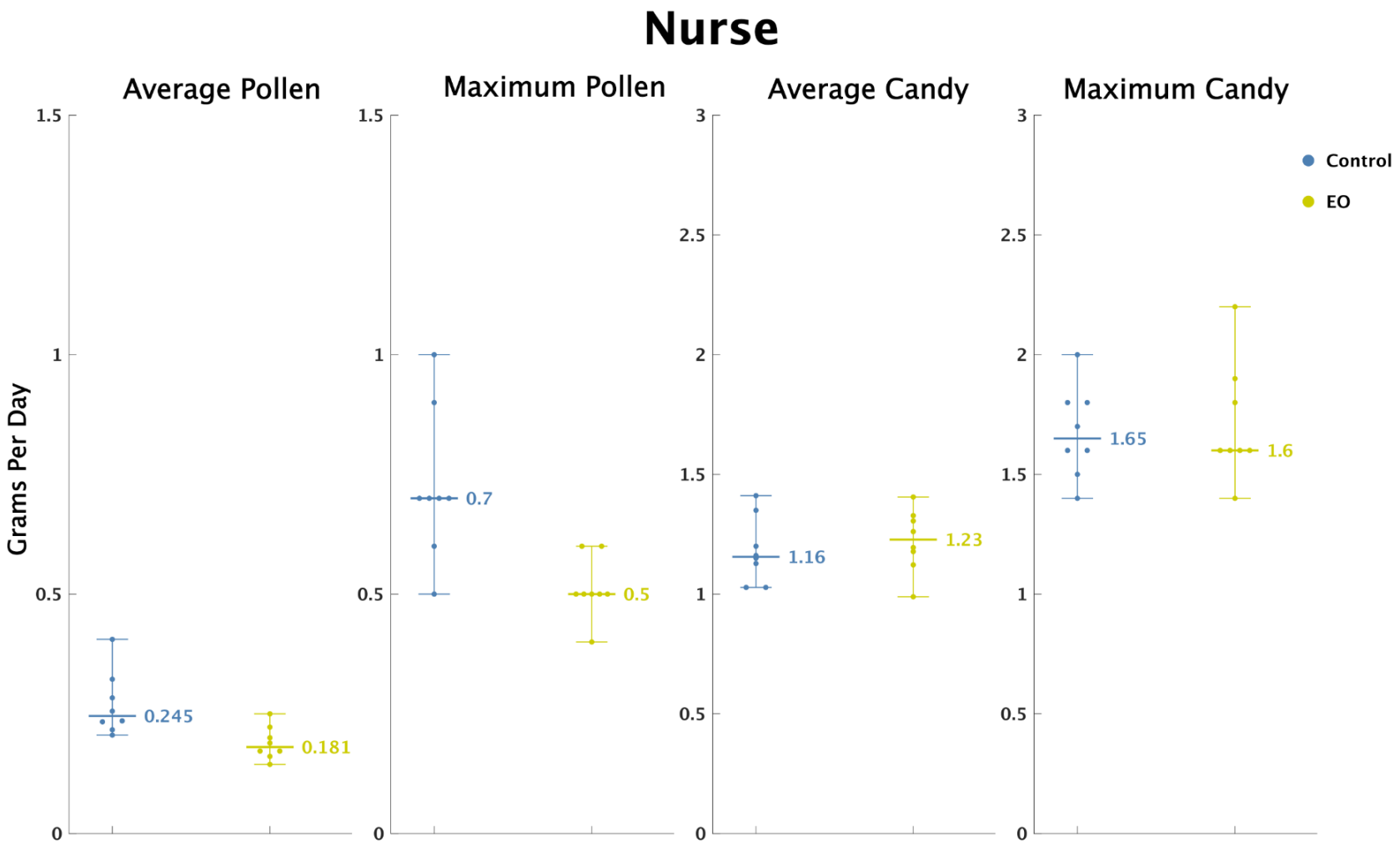

Figure 19. Daily food consumption for Nurse cages, with median values displayed. Average pollen p = 0.00451, maximum pollen p = 0.00389, average candy p = 0.624, maximum candy p = 0.898. N = 16: Control = 8, Ethyl Oleate (EO) = 8.

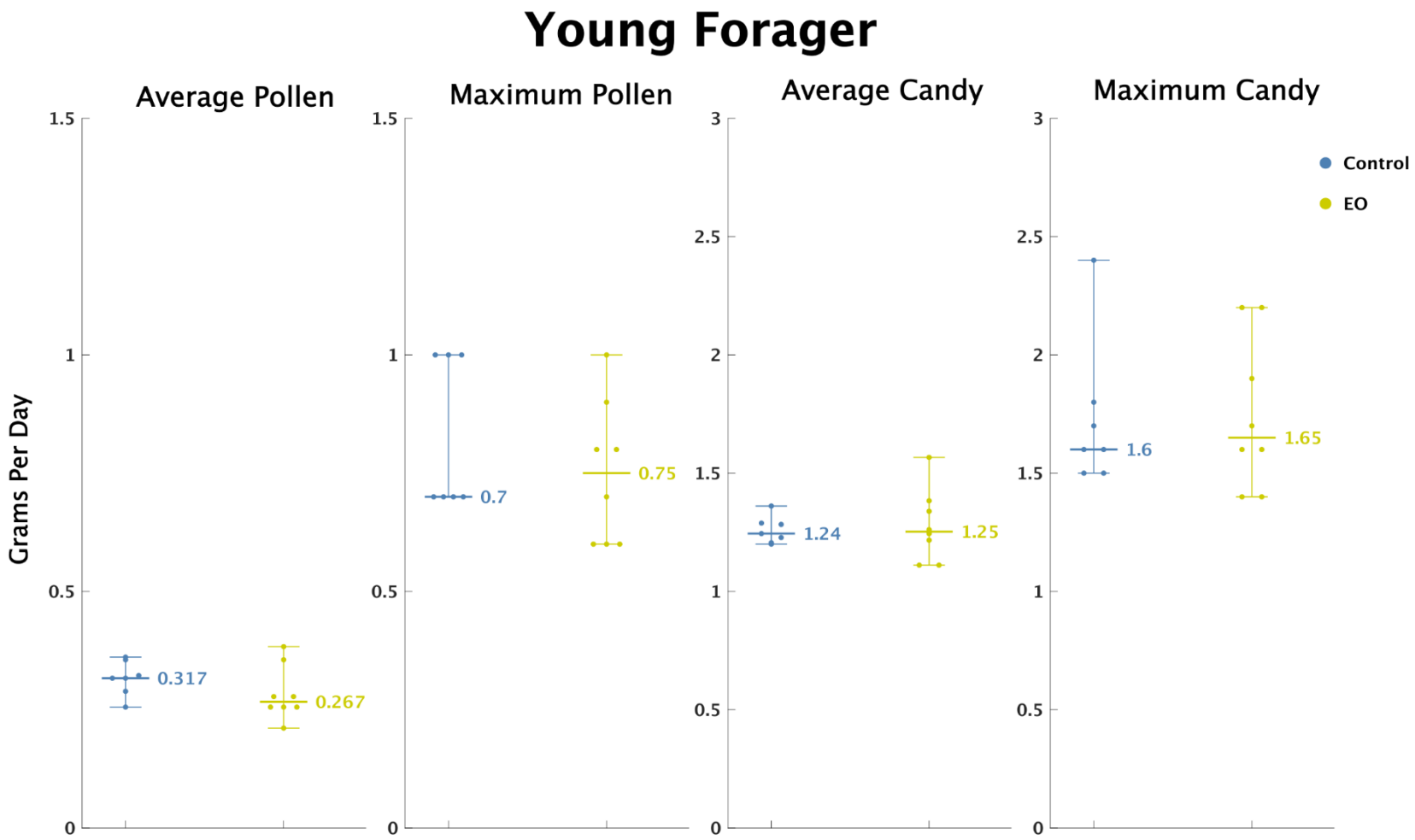

Figure 20. Daily food consumption for Young Forager (YF) cages, with median values displayed. Average pollen p = 0.145, maximum pollen p = 0.322, average candy p = 0.837, maximum candy p = 0.966. N = 15: Control = 7, Ethyl Oleate (EO)=8).

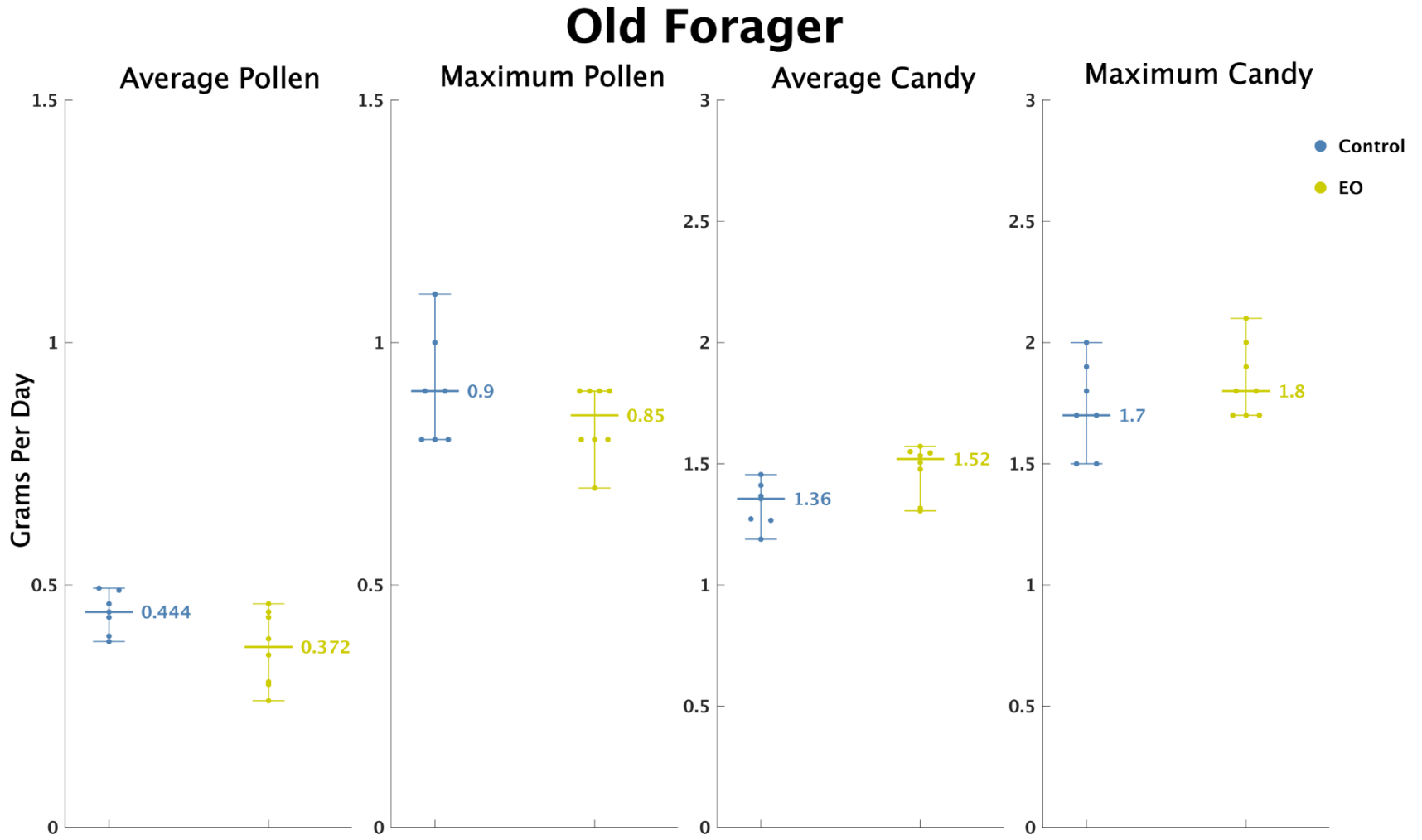

Figure 21. Daily food consumption for Old Forager (OF) cages, with median values displayed. Average pollen p = 0.0429, maximum pollen p = 0.427, average candy p = 0.0205, maximum candy p = 0.324. N = 15: Control = 7, Ethyl Oleate (EO) = 8.

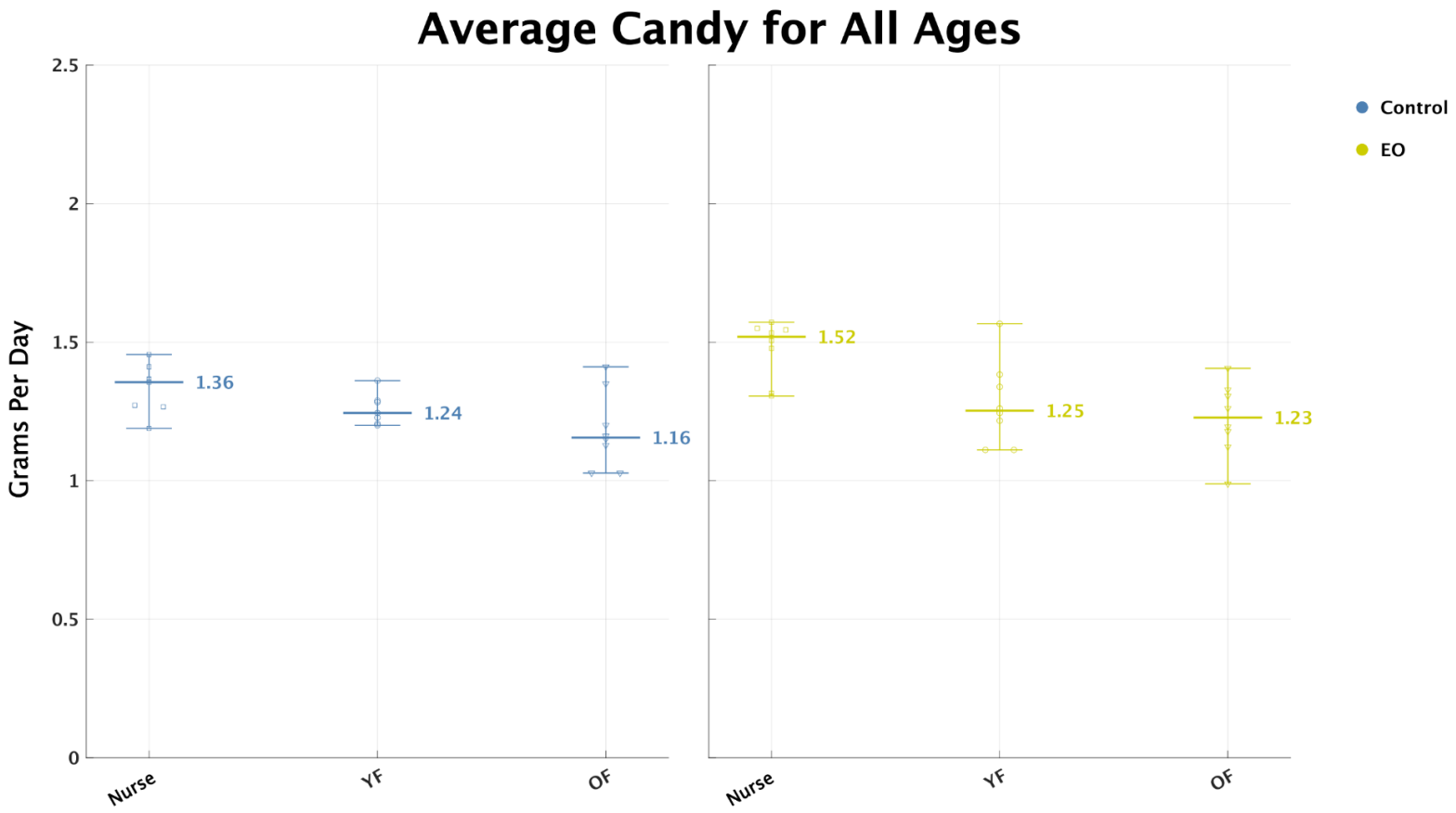

Figure 22. Average candy for all ages, with median values displayed. Control p= 0.0439 (Control N = 22: Nurse = 8, Young Forager (YF) = 7, Old Forager (OF) = 7). Ethyl Oleate (EO) p = 0.00924 (EO N = 24: Nurse = 8, YF = 8 , OF = 8).

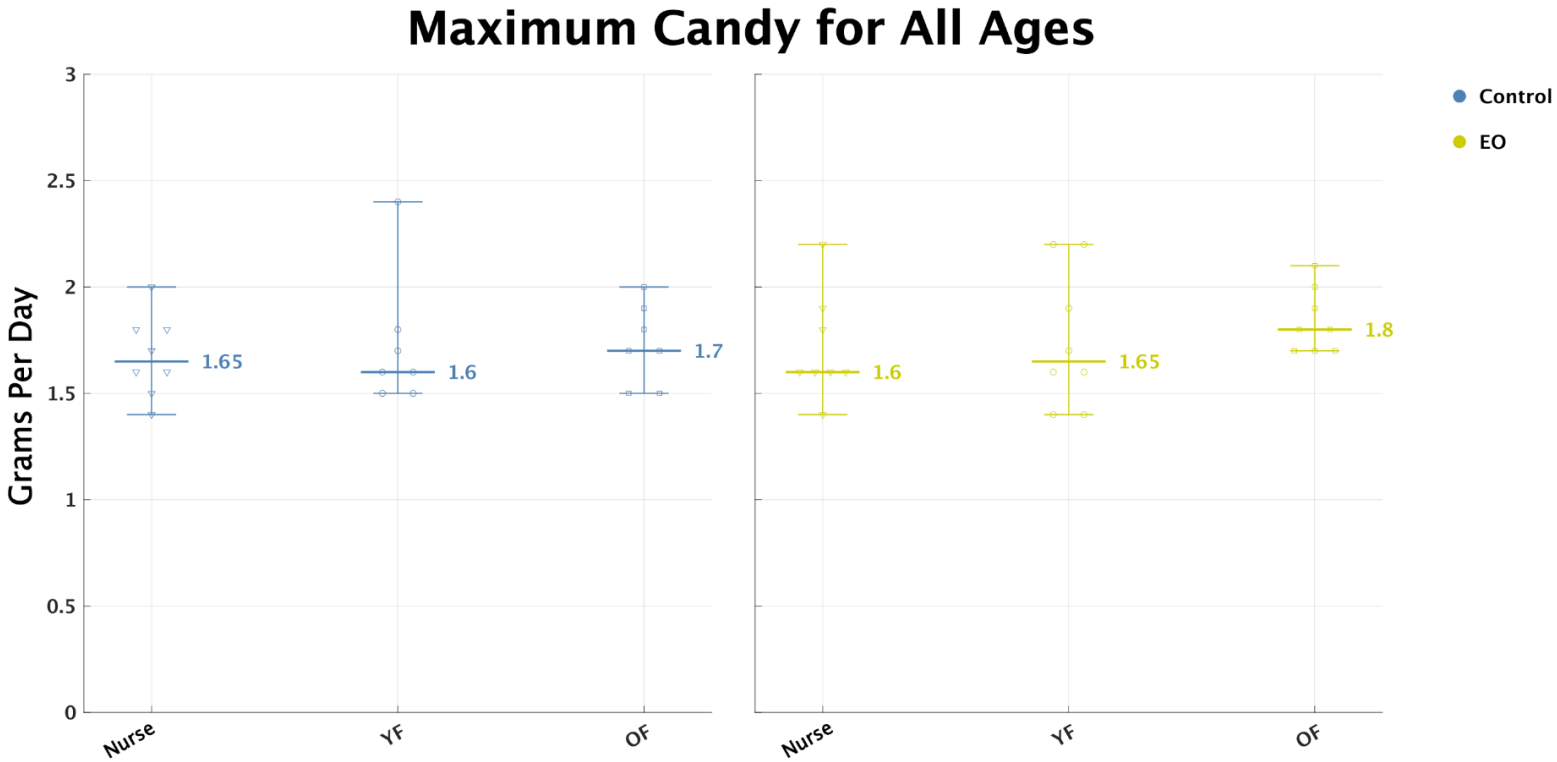

Figure 23. Maximum candy for all ages, with median values displayed. Control p = 0.83 (Control N = 22: Nurse = 7, Young Forager (YF) = 7, Old Forager (OF) = 8). Ethyl Oleate (EO) p = 0.33 (EO N = 24: Nurse = 8, YF = 8 , OF = 8).

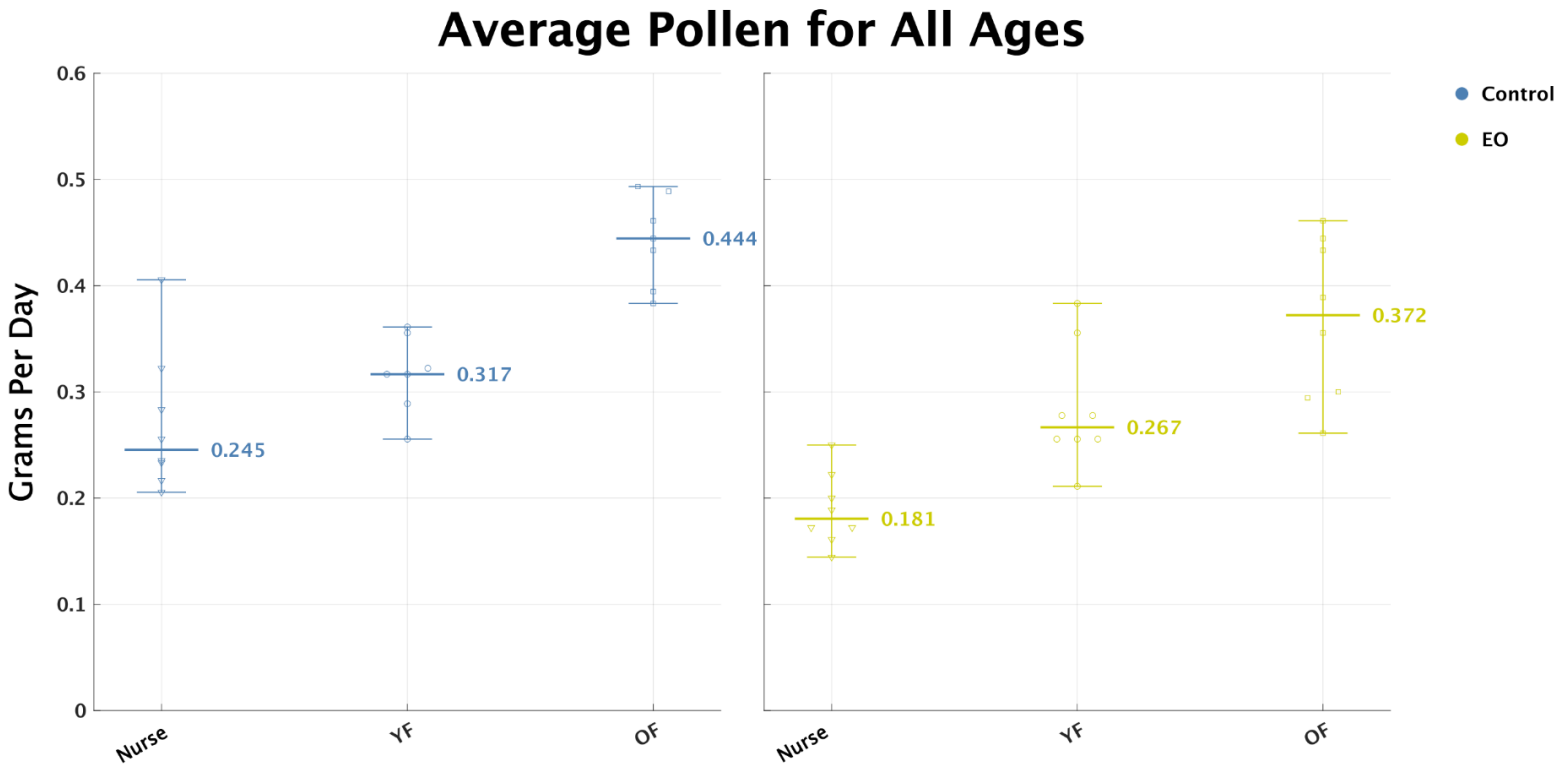

Figure 24. Average pollen for all ages, with median values displayed. Control p = 0.00103 (Control N = 22: Nurse = 8, Young Forager (YF) = 7, Old Forager (OF) = 7). Ethyl Oleate (EO) p = 0.000174 (EO N = 24: Nurse = 8, YF = 8 , OF = 8).

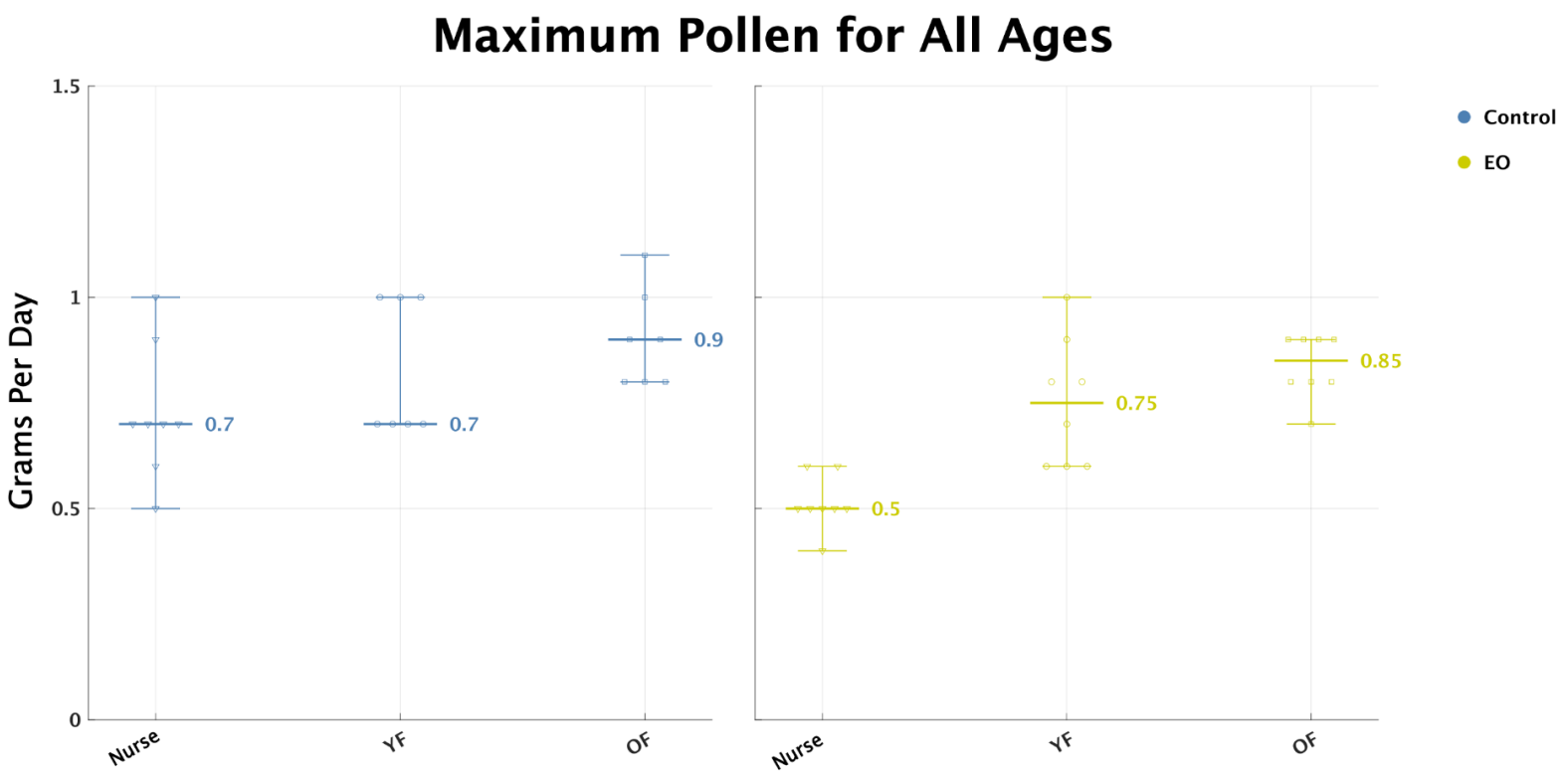

Figure 25. Maximum pollen for all ages, with median values displayed. Control p = 0.0734 (Control N = 22: Nurse = 8, Young Forager (YF) = 7, Old Forager (OF) = 7). Ethyl Oleate (EO) p = 0.000435 (EO N = 24: Nurse = 8, YF = 8 , OF = 8).

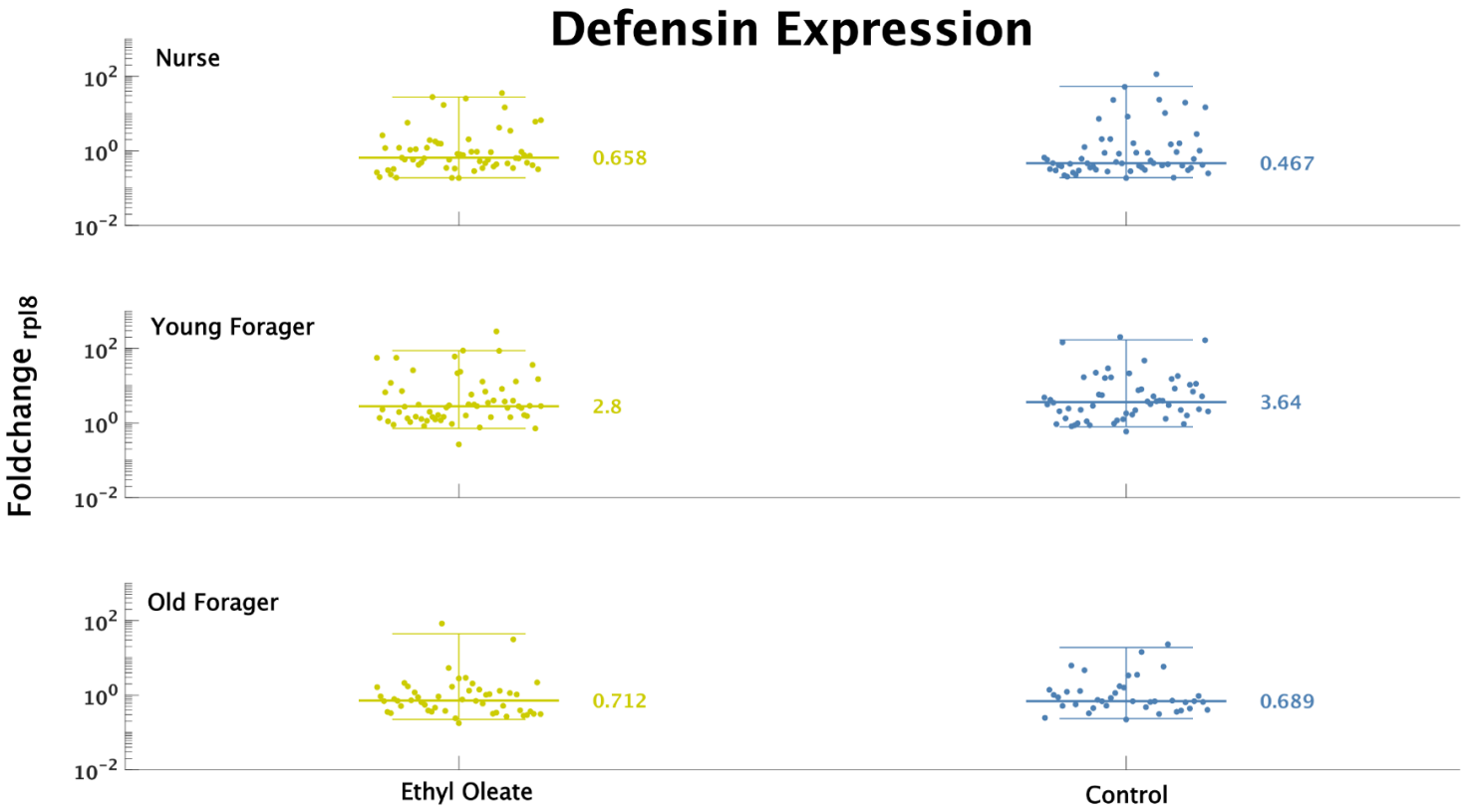

Figure 26. *Defensin* expression with *rpl8* as a reference gene, with median values displayed. Nurse p = 0.238 (N = 120: Ethyl Oleate (EO) = 61, Control = 59). Young Forager (YF) p = 0.443 (N = 117: EO = 61, Control = 56). Old Forager (OF) p = 0.565 (N= 89: EO = 50, Control = 39).

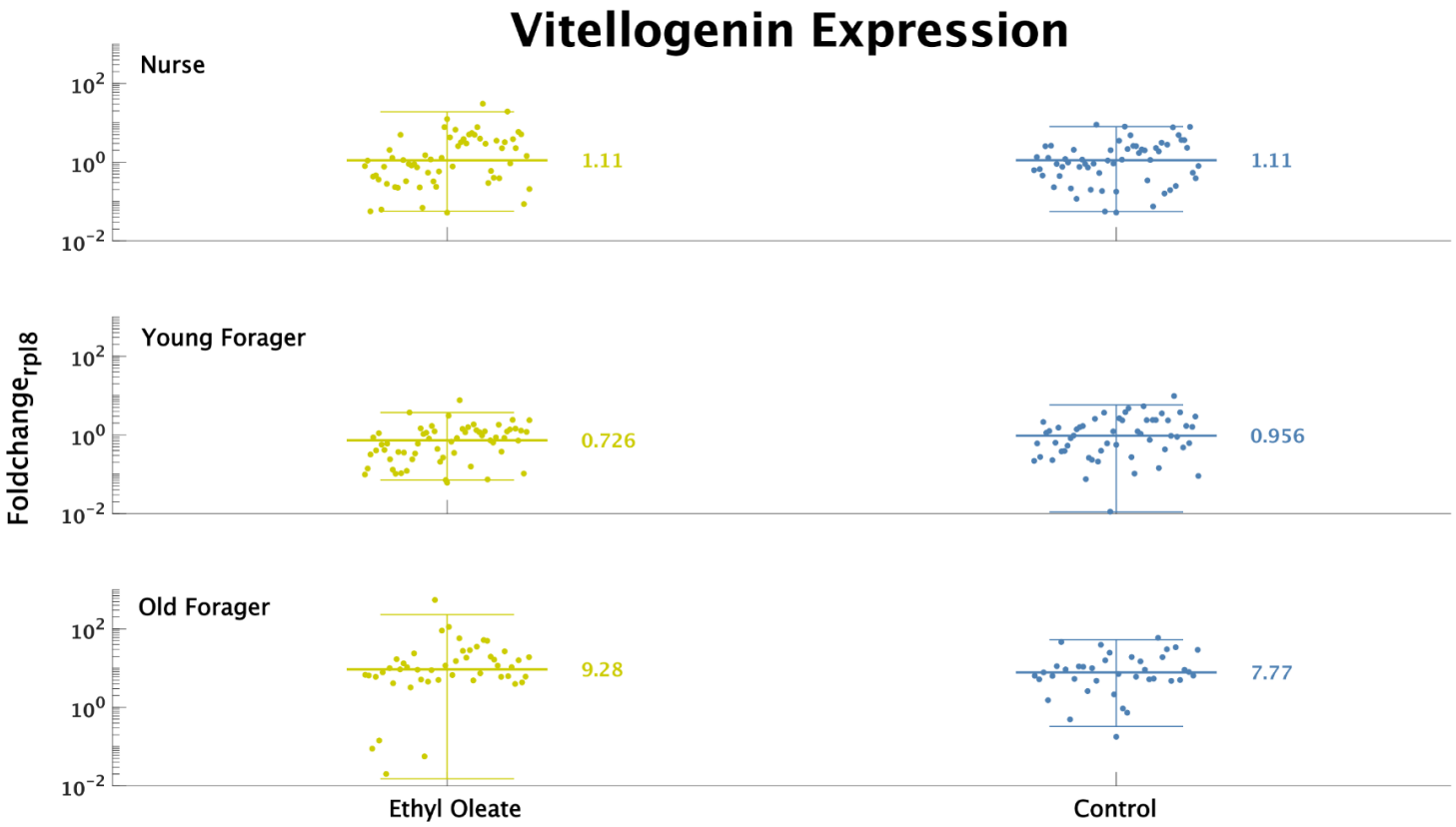

Figure 27. *Vitellogenin* expression with *rpl8* as a reference gene, with median values displayed. Nurse p = 0.527 (N = 128: (Ethyl Oleate (EO) = 62, Control = 66). Young Forager (YF) p = 0.1 (N= 118: EO = 61, Control = 57). Old Forager (OF) p = 0.425 (N = 89: EO = 50, Control = 39).

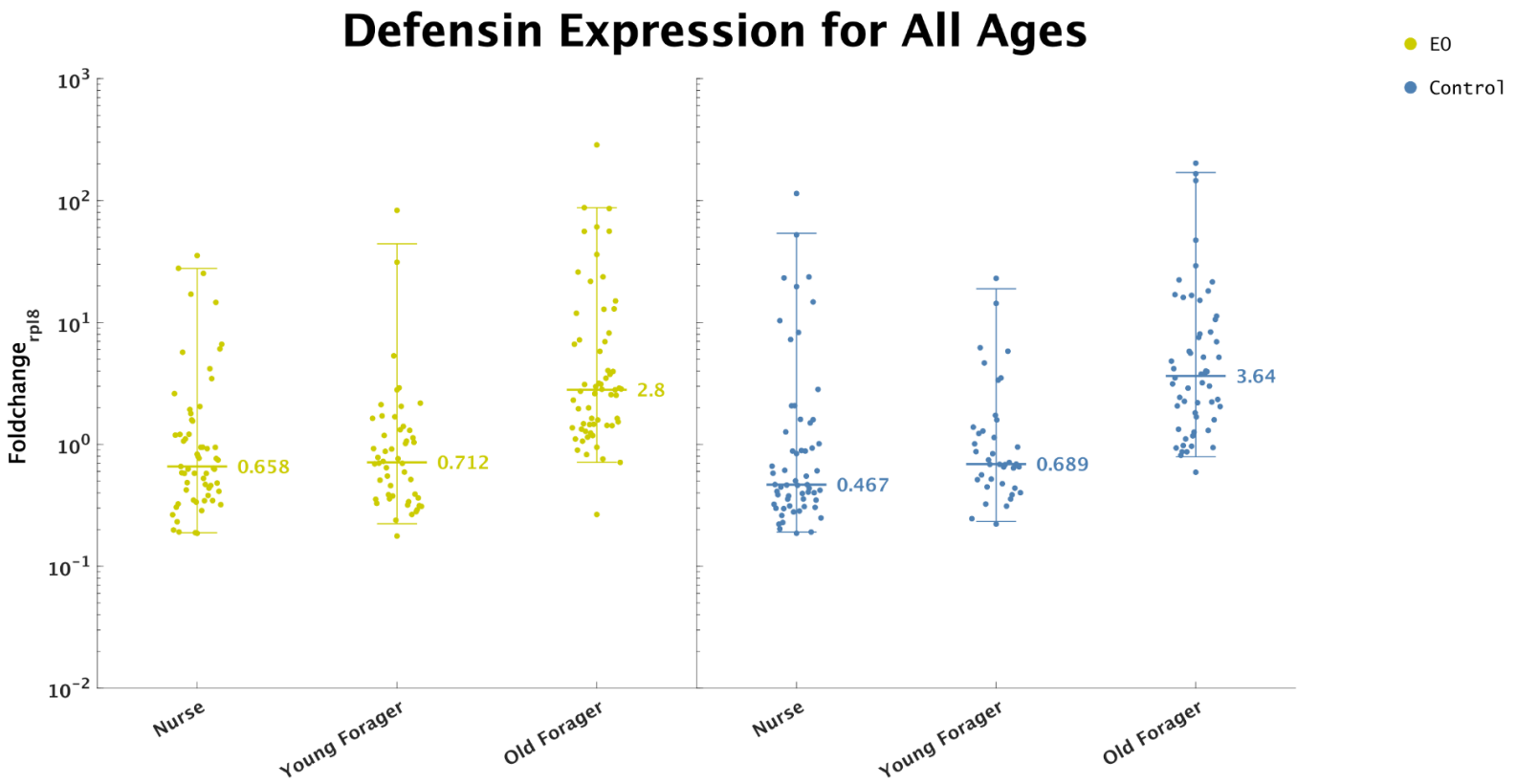

Figure 28. *Defensin* expression for all ages with *rpl8* as a reference gene, with median values displayed. Ethyl Oleate (EO) p= 2.21 x10^{-11} (N = 172: EO Nurse = 61, EO Young Forager (YF) = 61, EO Old Forager (OF) = 50). Control p = 3.73 x10^{-11} (N = 154: Control Nurse = 59, Control YF = 56, Control OF = 39).

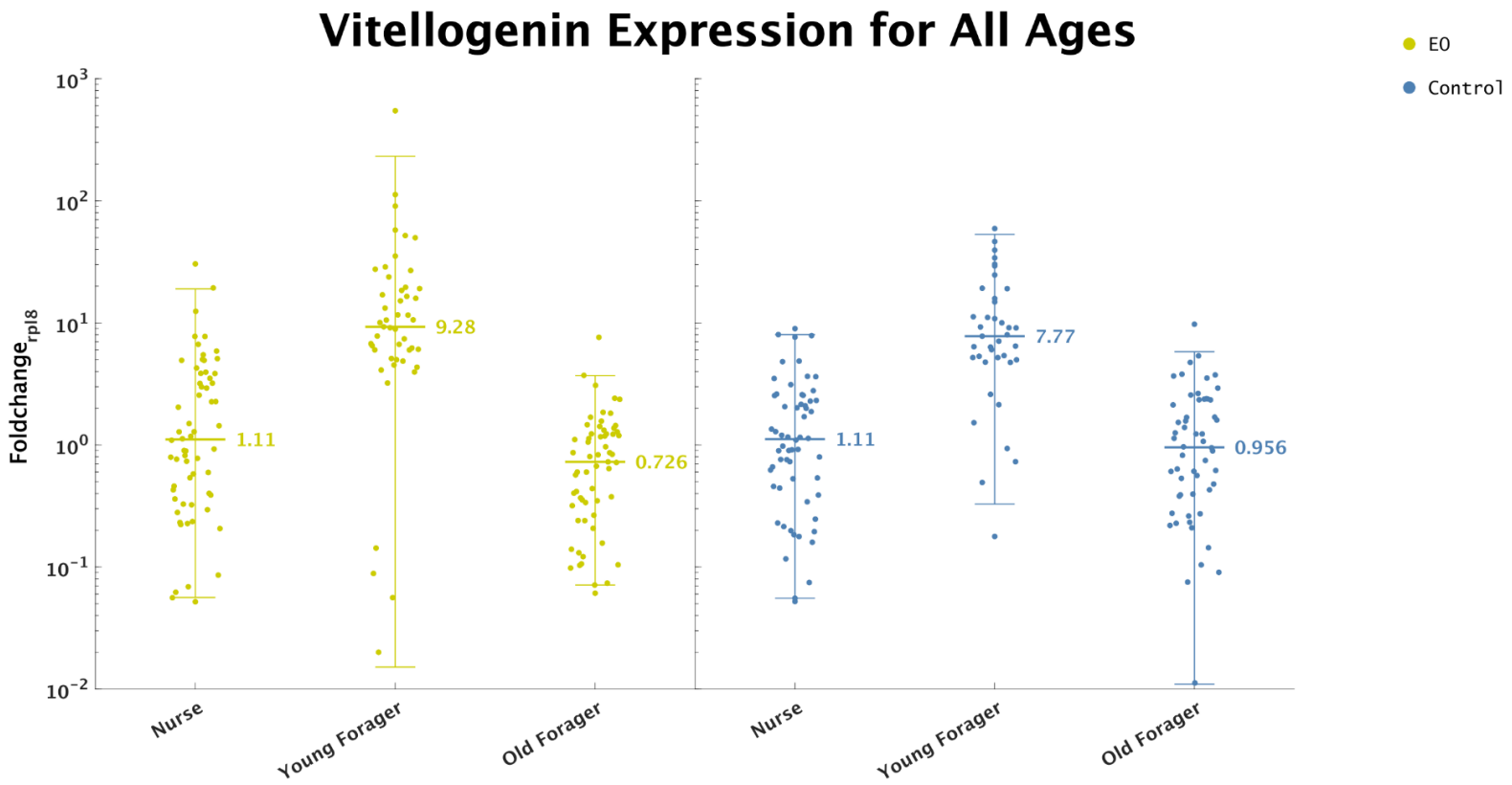

Figure 29. *Vitellogenin* expression for all ages with *rpl8* as a reference gene, with median values displayed. Ethyl Oleate (EO) p = 6.26 x10^{-14} (N = 173: EO Nurse = 62, EO Young Forager (YF) = 61, EO Old Forager (OF) = 50). Control p = 2.86 x10^{-12} (N = 156: Control Nurse= 66, Control YF = 57, Control OF = 39).

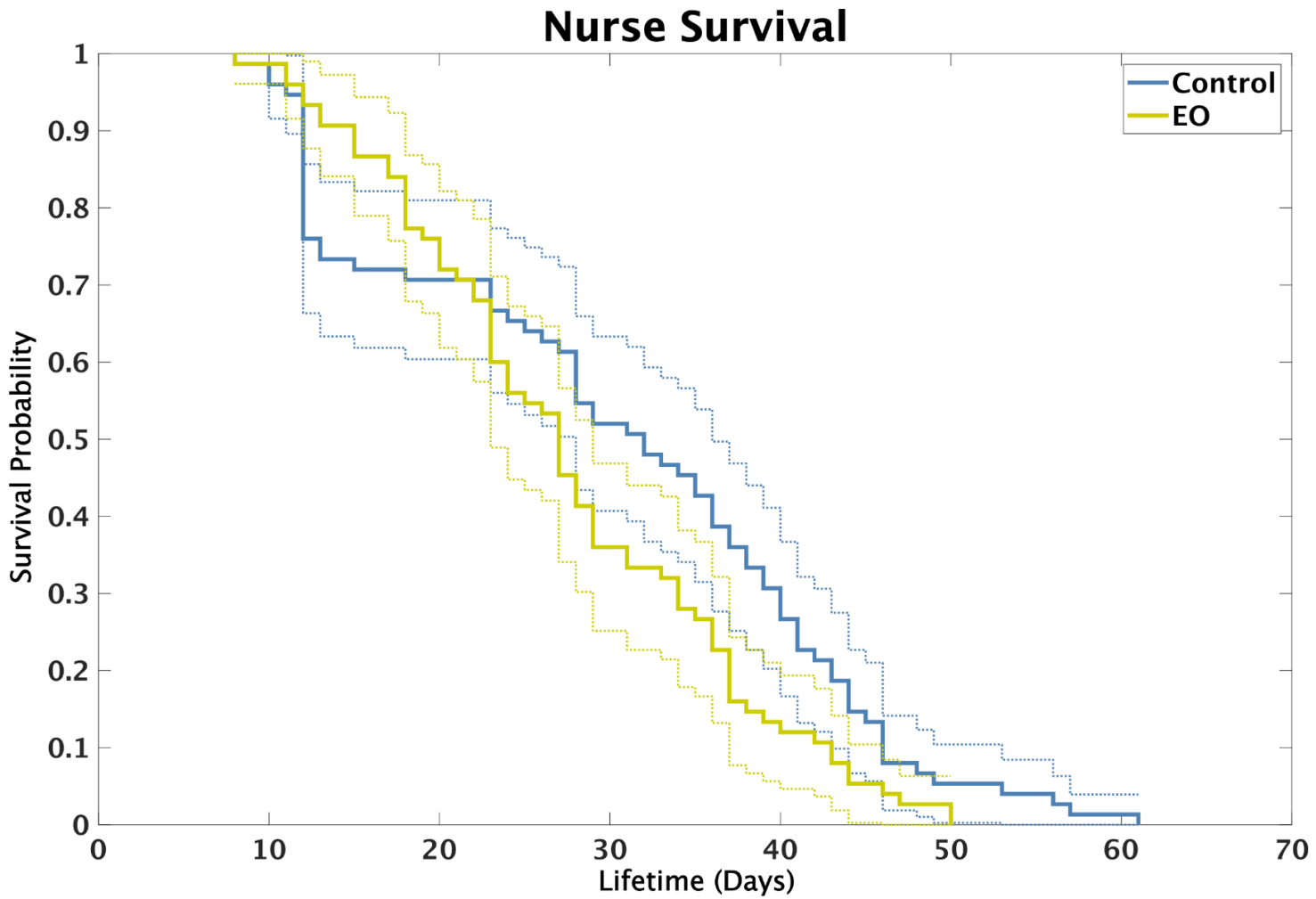

Figure 30. Cox proportional hazards regression model for Nurses. Dotted lines represent error margins. (p = 0.487; N = 150: Control = 75, Ethyl Oleate (EO) = 75).

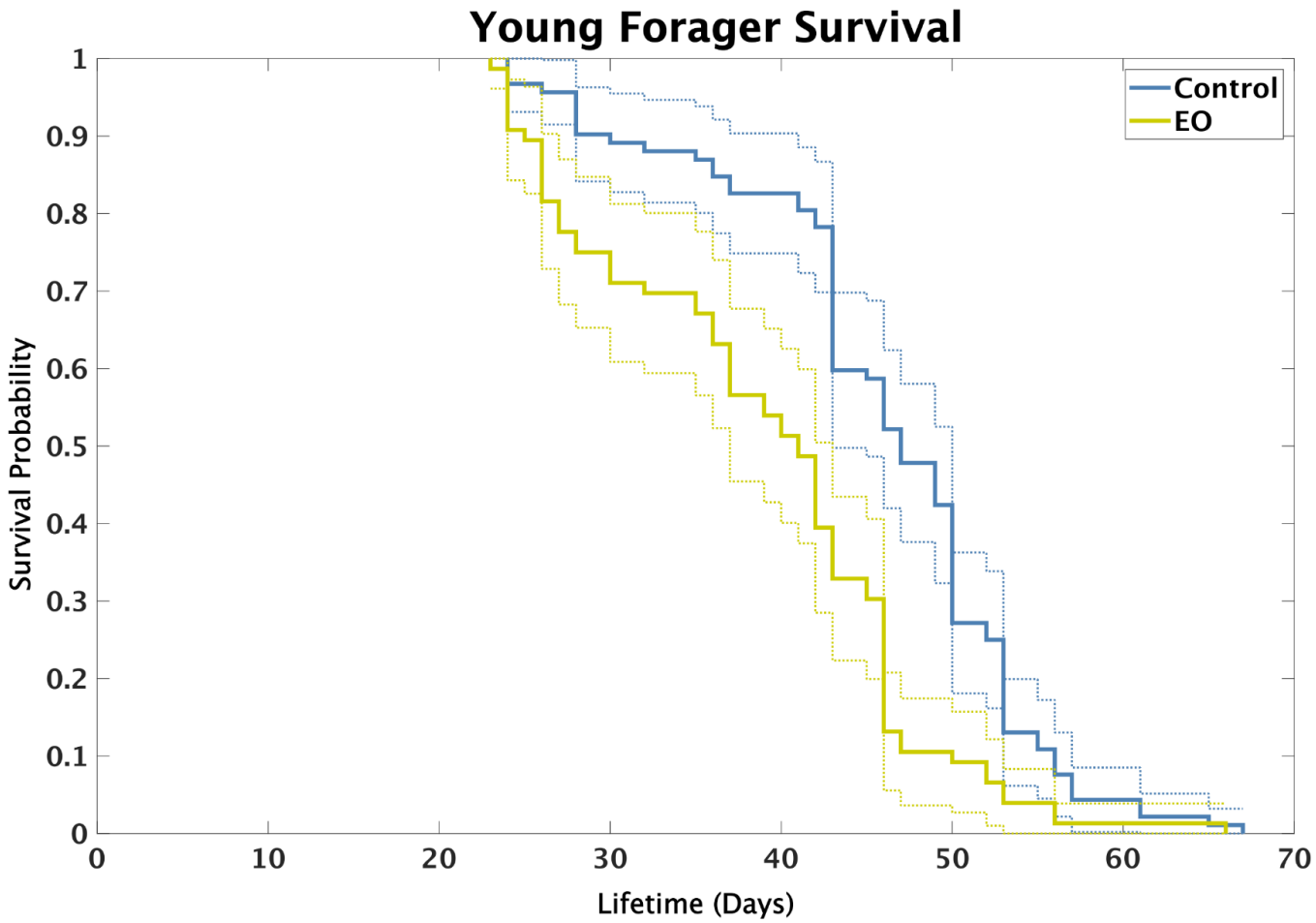

Figure 31. Cox proportional hazards regression model for Young Foragers (YF). Dotted lines represent error margins. (p = 0.0183; N = 168: Control = 92, Ethyl Oleate (EO) = 76).

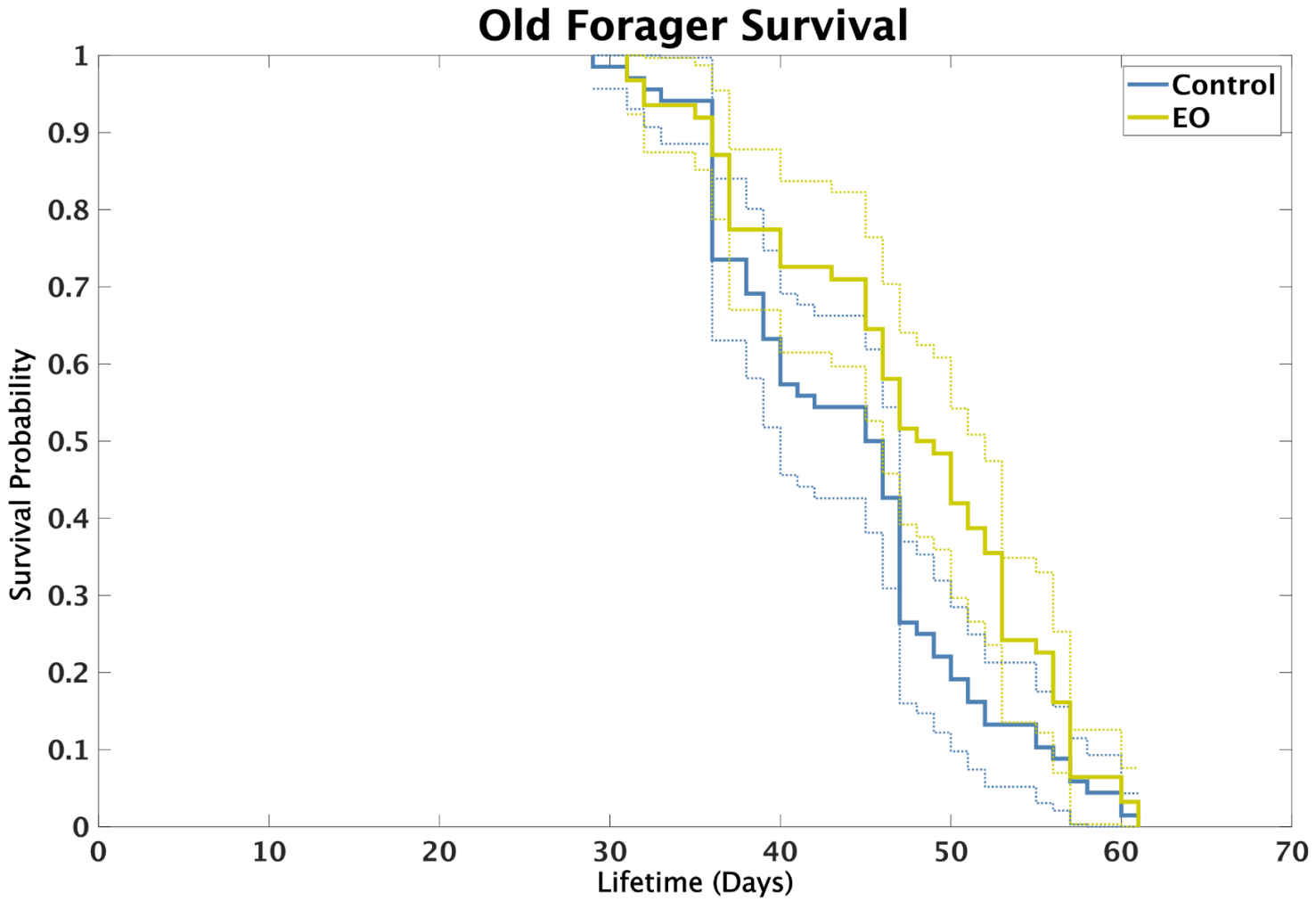

Figure 32. Cox proportional hazards regression model for Old Foragers (OF). Dotted lines represent error margins. (p = 0.24; N = 131: Control = 68, Ethyl Oleate (EO) = 63).

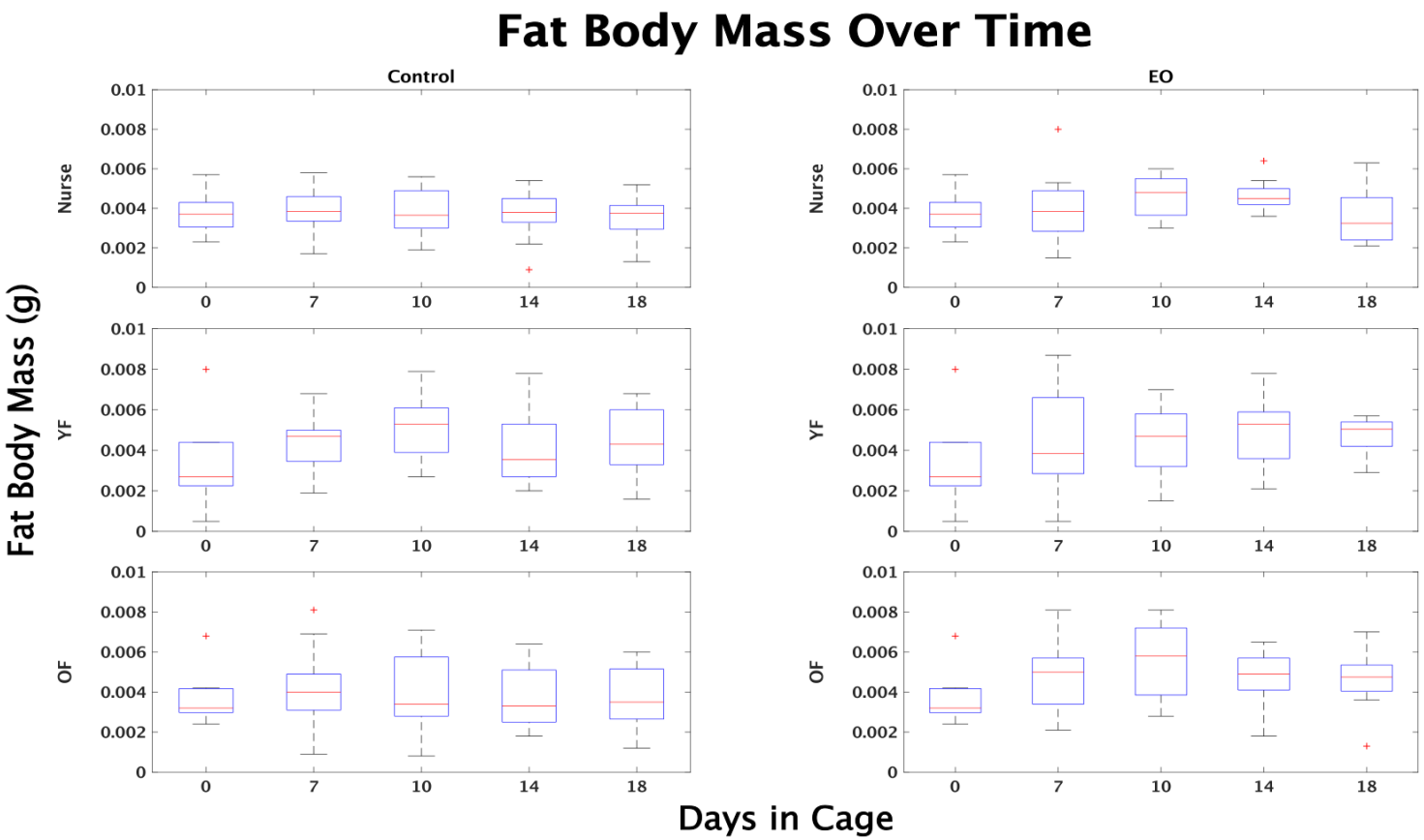

Figure 33. Boxplots for fat body mass for each sampling day (not pooled). The "baseline" bees are represented by "Day 0" and the baseline bees are the same for both Ethyl Oleate (EO) treatment and Control. N = 366: Control Nurse = 62, Ethyl Oleate (EO) Nurse = 64, baseline Nurse = 8, Control Young Forager (YF) = 57, EO YF = 63, baseline YF = 7, Control Old Forager (OF) = 47, EO OF = 51, baseline OF = 7.

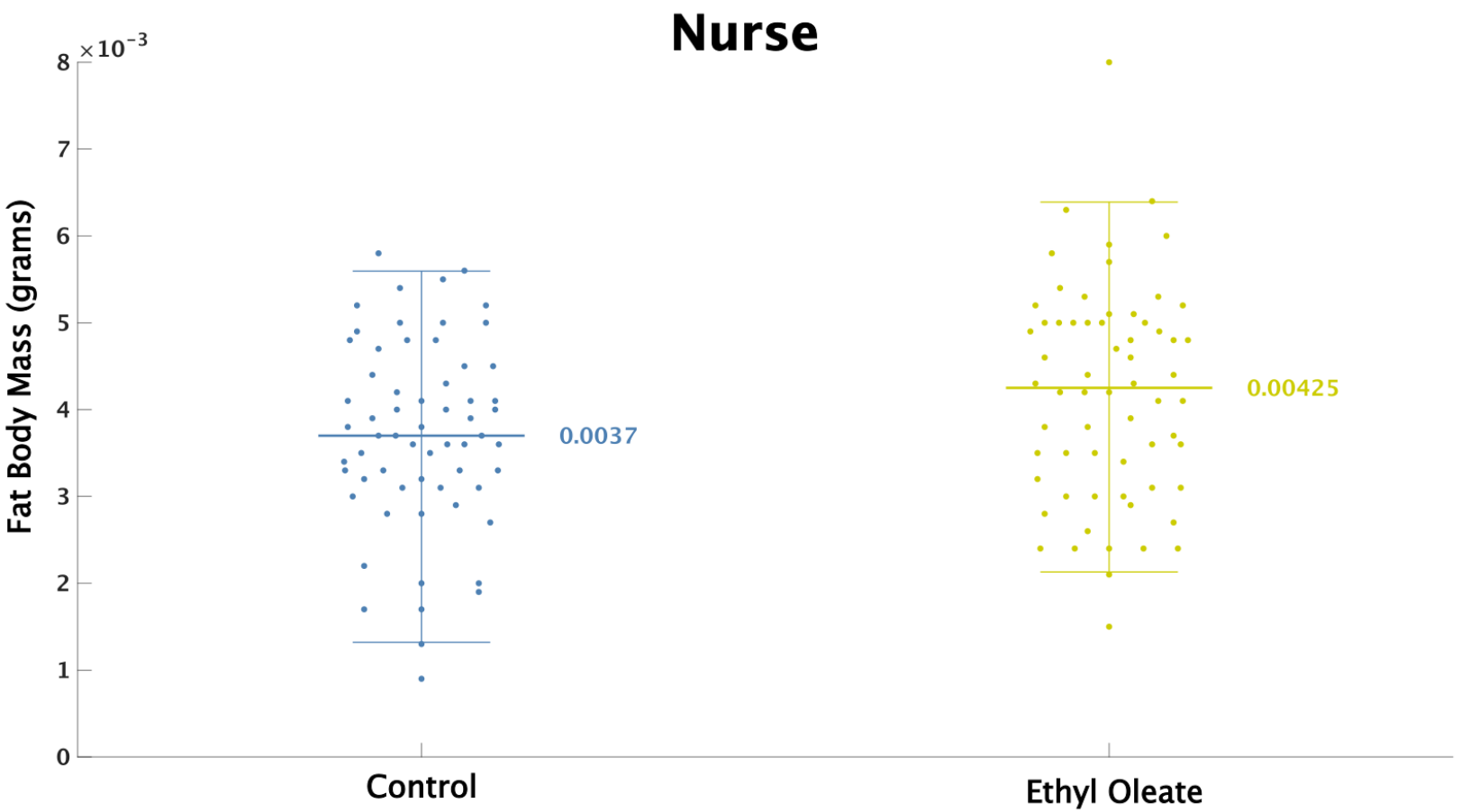

Figure 34. Fat body mass for nurses, with median values displayed. Wilcoxon-Mann-Whitney p = 0.041 (N = 126: Control = 62, EO = 64).

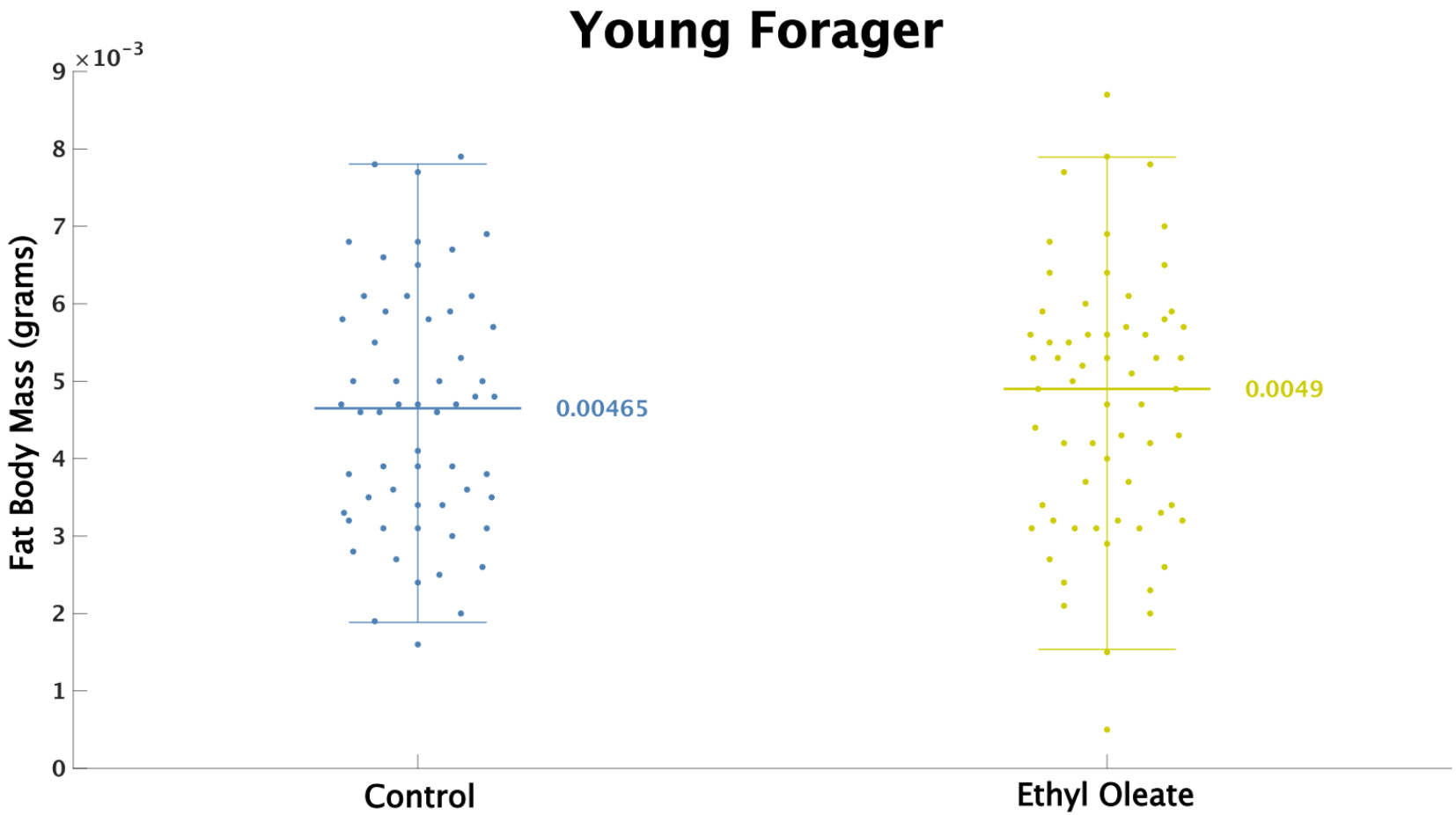

Figure 35. Fat body mass for young foragers, with median values displayed. Wilcoxon-Mann-Whitney p = 0.659 (N = 121: Control = 58, EO = 63).

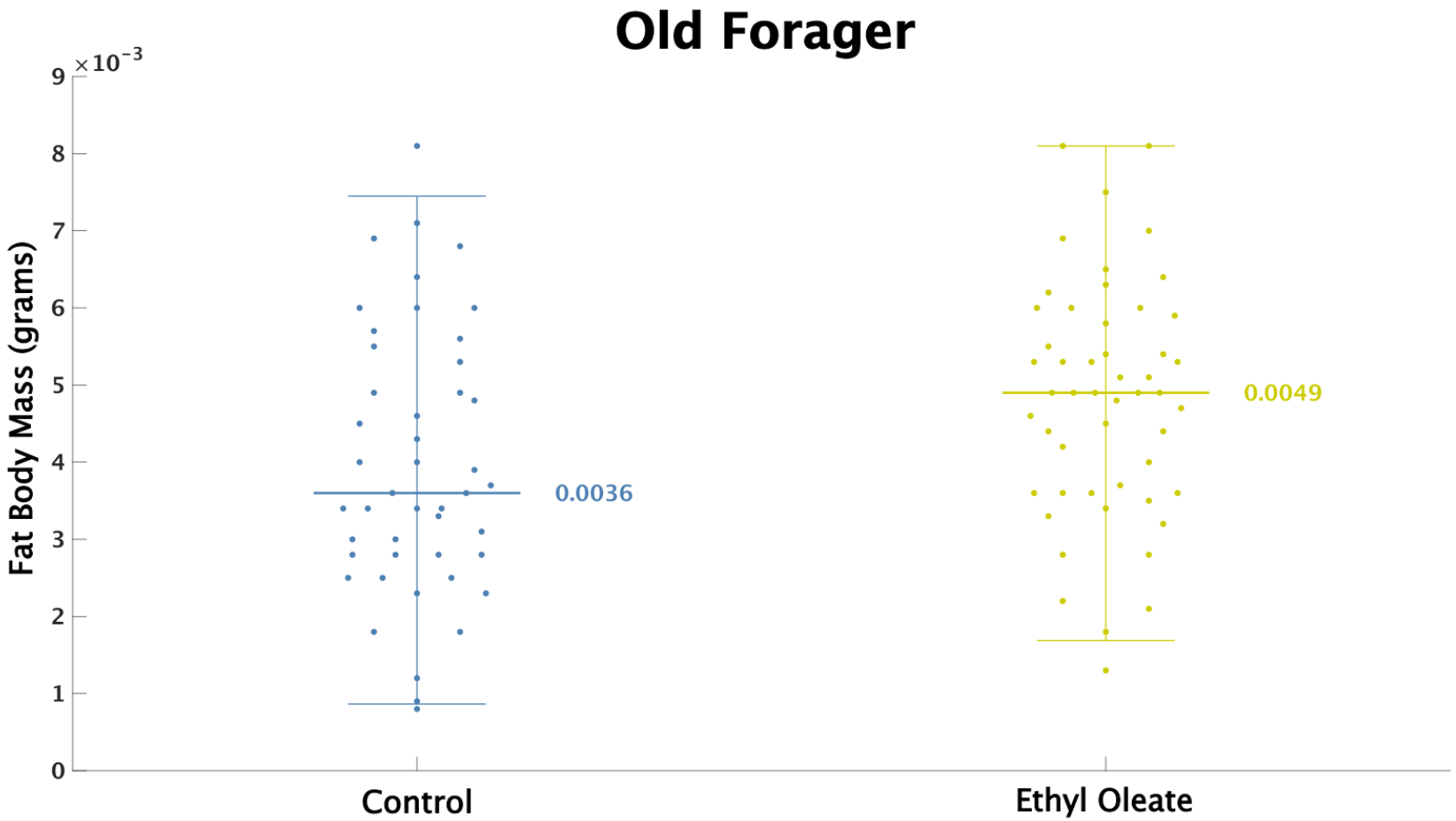

Figure 36. Fat body mass for old foragers, with median values displayed. Wilcoxon-Mann-Whitney p = 0.01 (N = 97: Control = 46, EO = 51).

Figure 37. Fat body mass for all ages, with median values displayed. 3-way Kruskal-Wallis Control p = 0.0175 (Control N = 155: Nurse = 62, Young Forager (YF) = 58, Old Forager (OF) = 46). 3-way Kruskal-Wallis Ethyl Oleate (EO) p = 0.0342 (N = 178: Nurse = 64, YF = 63, OF = 51).

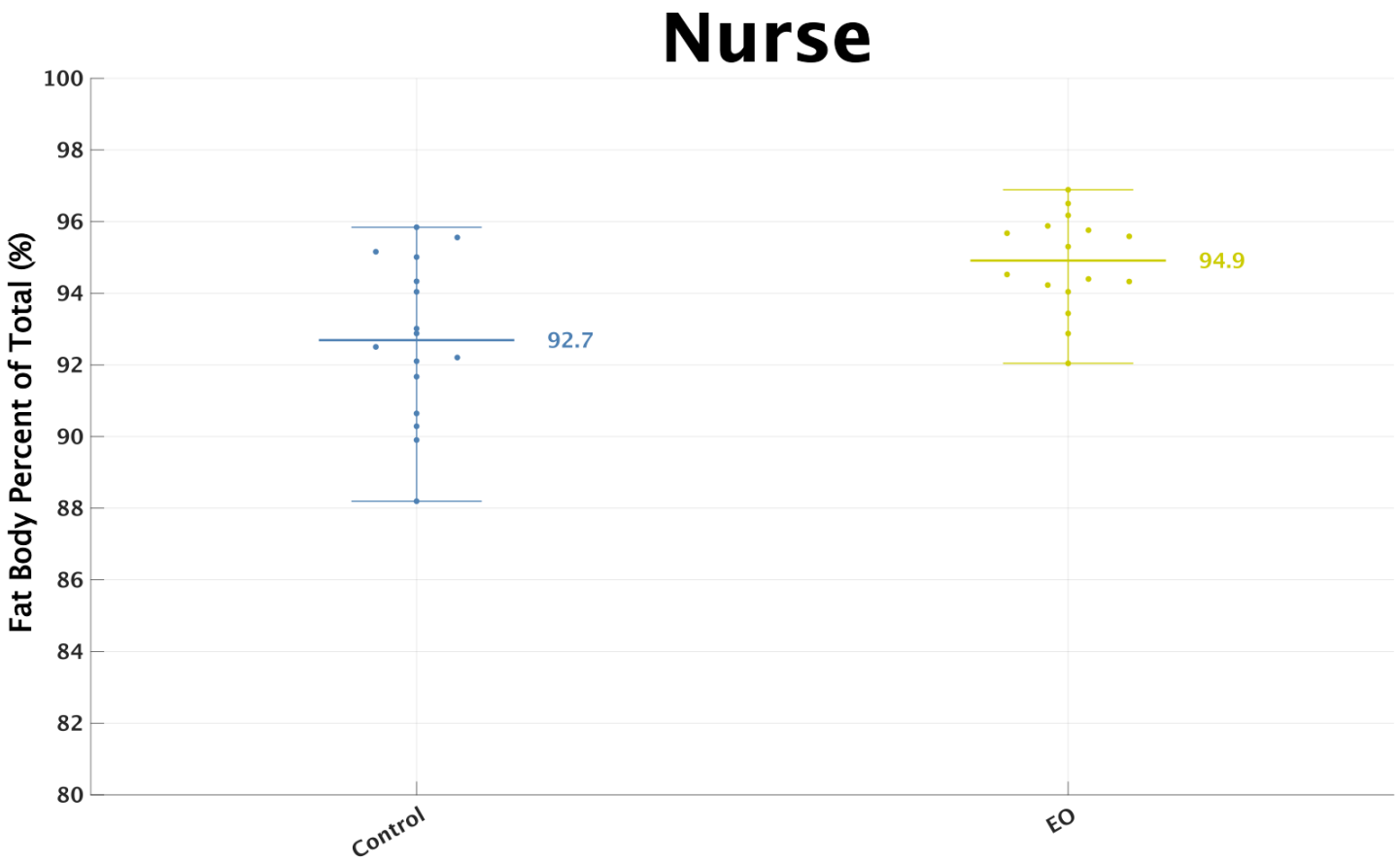

Figure 38. Fat body mass percentage of total (sum of fat body mass and HPG protein mass) for nurses, with median values displayed. Wilcoxon-Mann-Whitney p = 0.00499 (N = 32: Control = 16, Ethyl Oleate (EO) = 16).

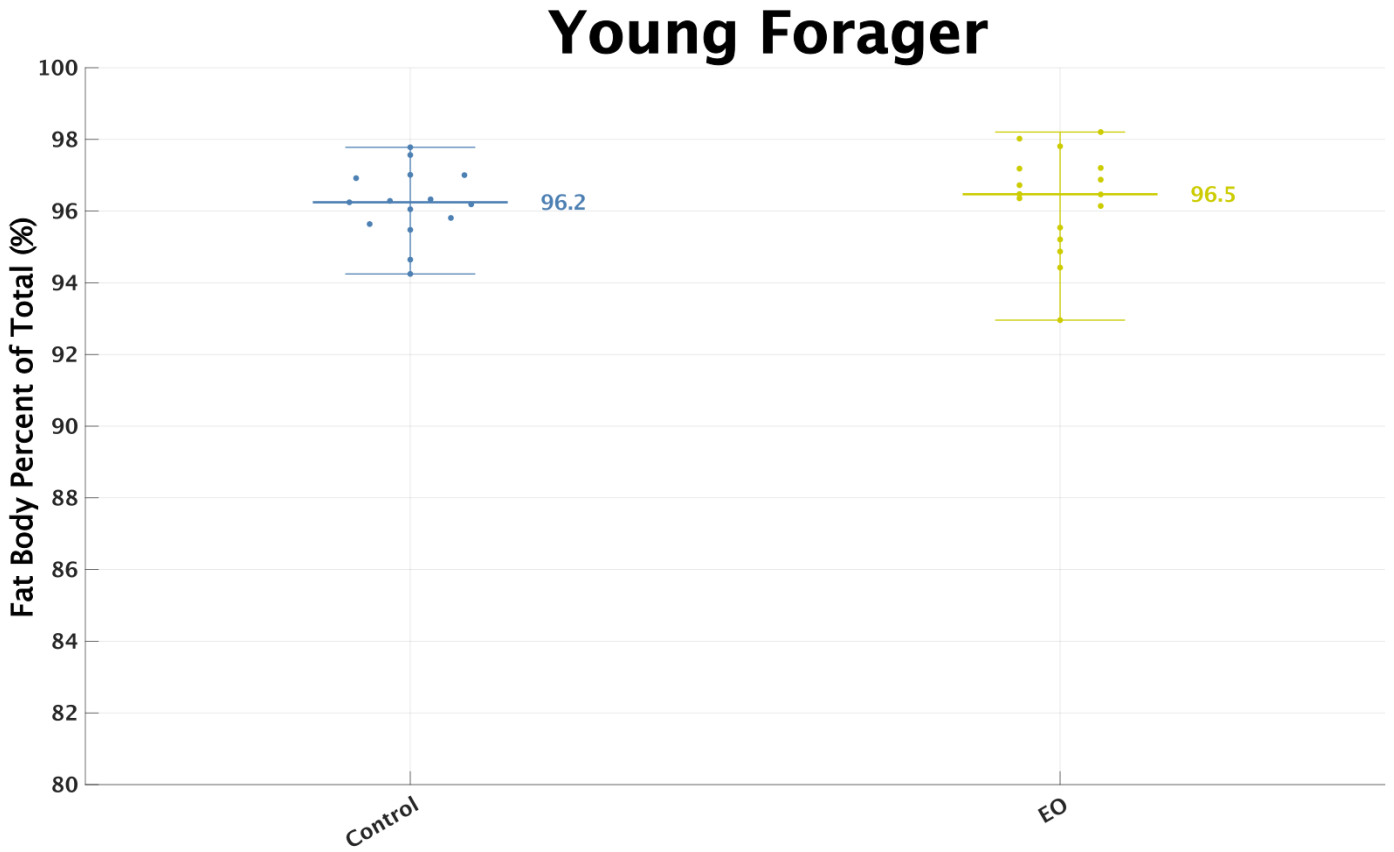

Figure 39. Fat body mass percentage of total (sum of fat body mass and HPG protein mass) for young foragers, with median values displayed. Wilcoxon-Mann-Whitney p = 0.567 (N = 31: Control = 15, Ethyl Oleate (EO) =16).

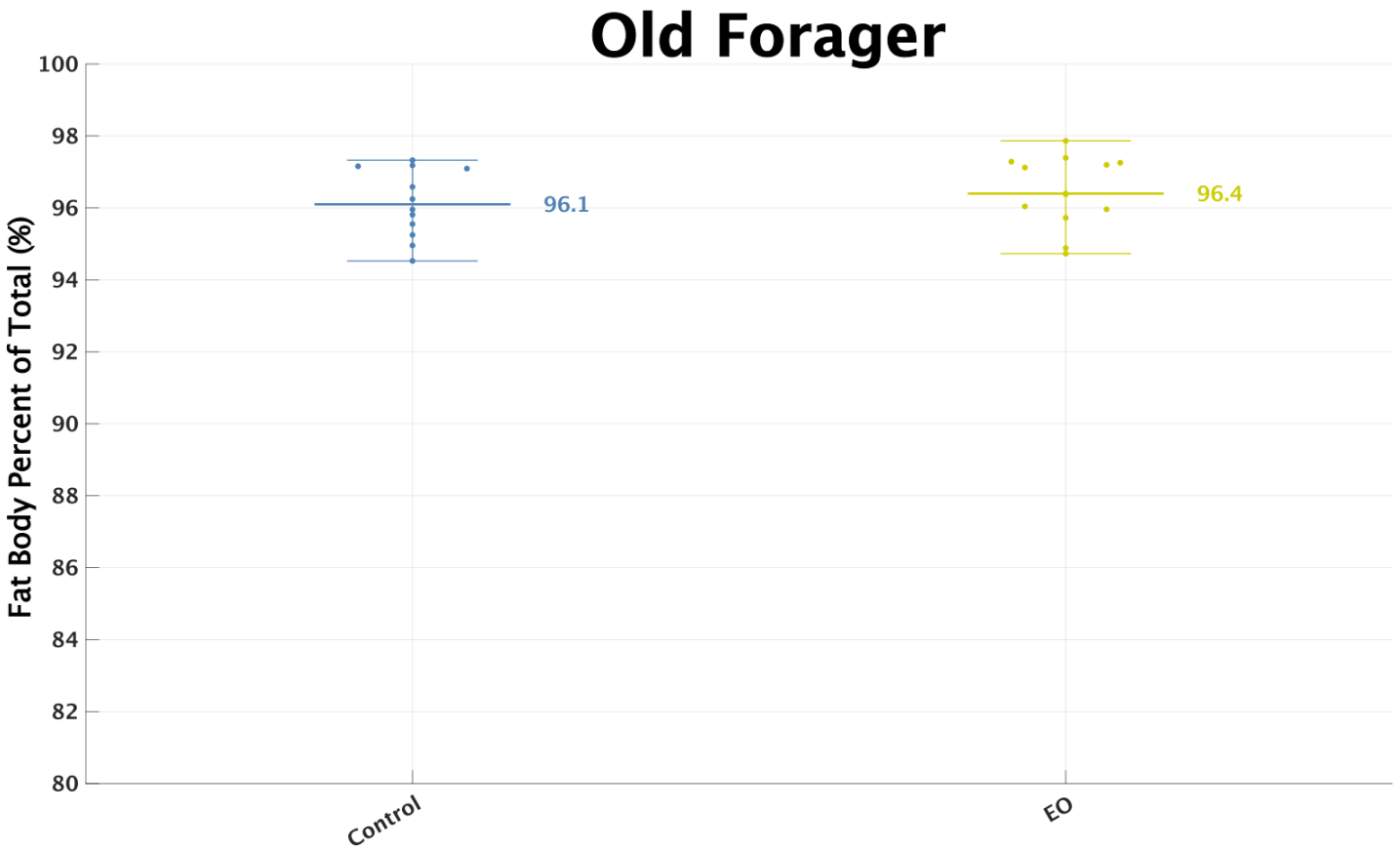

Figure 40. Fat body mass percentage of total (sum of fat body mass and HPG protein mass) for old foragers, with median values displayed. Wilcoxon-Mann-Whitney p = 0.289 (N = 25: Control = 12, Ethyl Oleate (EO) =13).

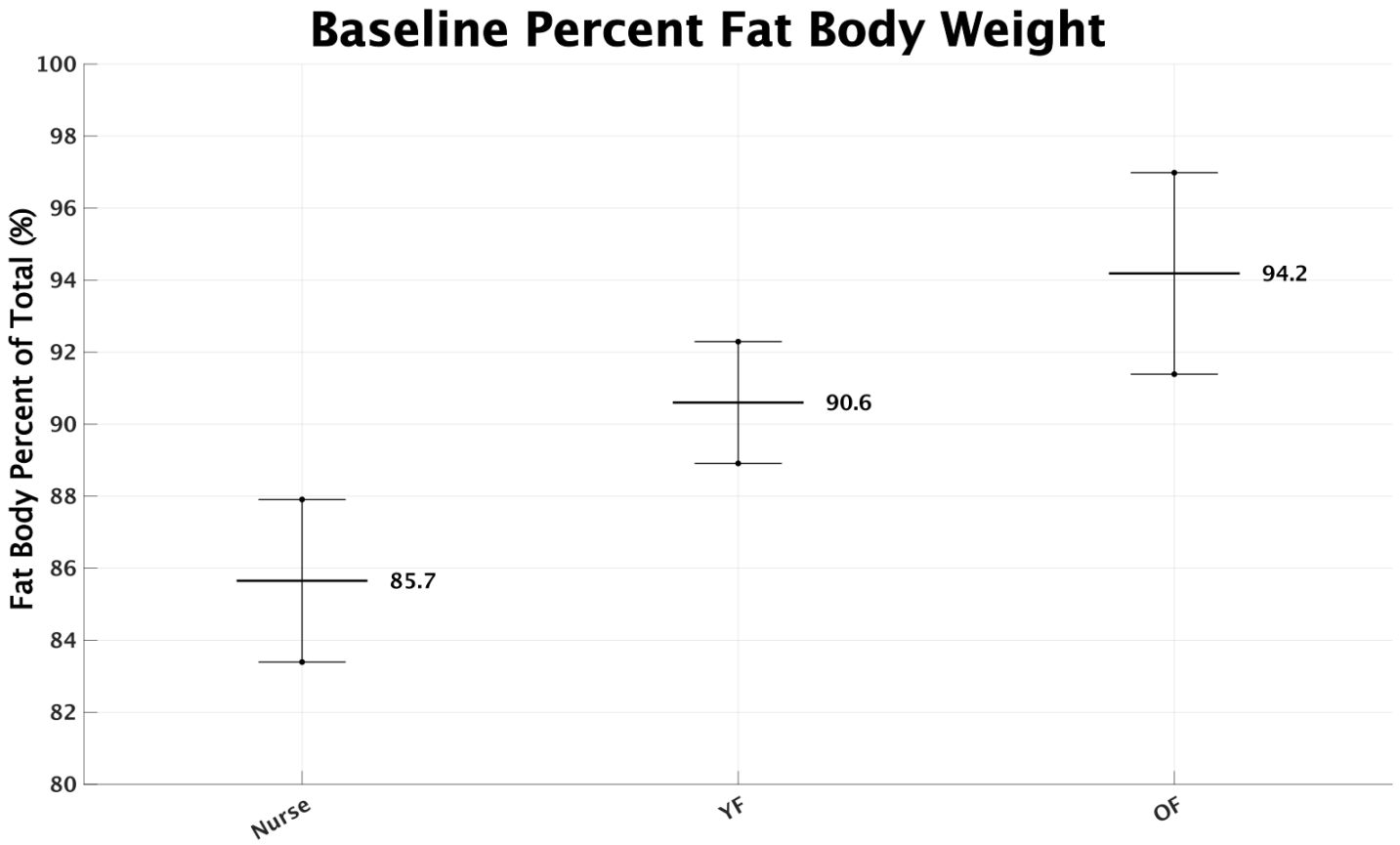

Figure 41. Fat body mass percentage of total (sum of fat body mass and HPG protein mass) for "baseline" bees. N = 2 for each age group (each data point represents 4 pooled bees).

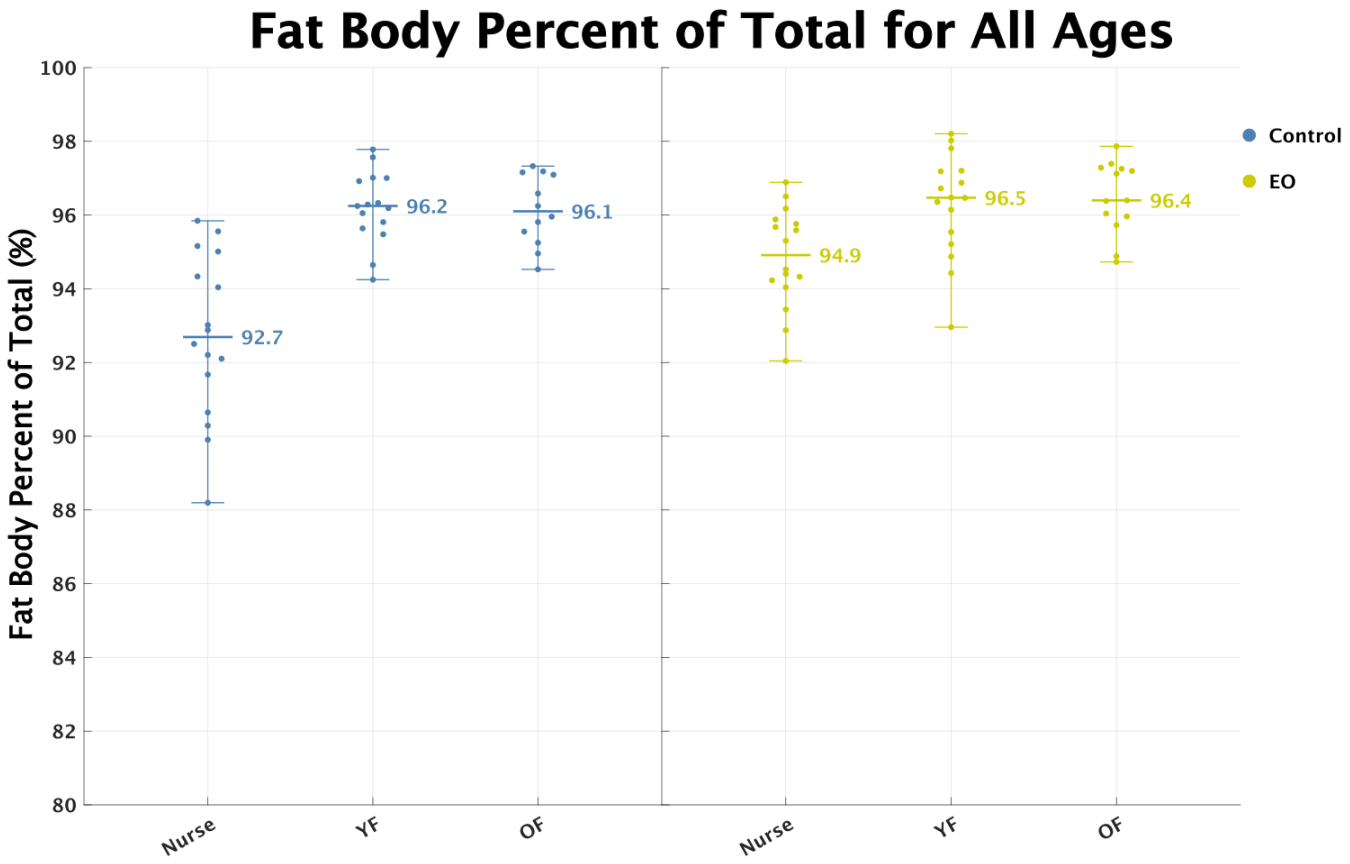

Figure 42. Fat body mass percentage of total (sum of fat body mass and HPG protein mass) for all ages, with median values displayed. Kruskal-Wallis Control p = 1.12 x10^{-05} (N = 43), Kruskal-Wallis Ethyl Oleate (EO) p = 0.0027 (N= 45).

References

1. Allen, M. D., & Jeffree, E. P. (1956). The influence of stored pollen and of colony size on the brood rearing of honeybees. *Annals of Applied Biology*, *44*(4), 649-656.
2. Amdam, G. V., & Omholt, S. W. (2002). The regulatory anatomy of honeybee lifespan. *Journal of theoretical biology*, *216*(2), 209-228.
3. Amdam, G. V., Norberg, K., Fondrk, M. K., & Page, R. E. (2004). Reproductive ground plan may mediate colony-level selection effects on individual foraging behavior in honey bees. *Proceedings of the National Academy of Sciences*, 101(31), 11350-11355.
4. Amdam, G. V., Aase, A. L. T., Seehuus, S. C., Fondrk, M. K., Norberg, K., & Hartfelder, K. (2005). Social reversal of immunosenescence in honey bee workers. *Experimental gerontology*, *40*(12), 939-947.
5. Brouwers, E. V. M. (1983). Activation of the hypopharyngeal glands of honeybees in winter. *Journal of Apicultural Research*, *22*(3), 137-141.
6. Bruckner, S., Steinhauer, N., Engelsma, J., Fauvel, A. M., Kulhanek, K., Malcolm, E., ... & Allendale, M. I. (2020). 2019–2020 Honey bee colony losses in the United States: preliminary results.
7. Casteels, P., Ampe, C., Jacobs, F., & Tempst, P. (1993). Functional and chemical characterization of Hymenoptaecin, an antibacterial polypeptide that is infection-inducible in the honeybee (Apis mellifera). *Journal of Biological Chemistry*, 268(10), 7044-7054.
8. Castillo, C., Chen, H., Graves, C., Maisonnasse, A., Le Conte, Y., & Plettner, E. (2012). Biosynthesis of ethyl oleate, a primer pheromone, in the honey bee (Apis mellifera L.). *Insect biochemistry and molecular biology*, *42*(6), 404-416.
9. Castillo, C., Maisonnasse, A., Le Conte, Y., & Plettner, E. (2012). Seasonal variation in the titers and biosynthesis of the primer pheromone ethyl oleate in honey bees. Jo*urnal of insect physiology*, 58(8), 1112-1121.
10. Chen, Y. P., Higgins, J. A., & Feldlaufer, M. F. (2005). Quantitative real-time reverse transcription-PCR analysis of deformed wing virus infection in the honeybee (Apis mellifera L.). *Applied and Environmental Microbiology*, 71(1), 436-441.
11. Collins, A. M., Williams, V., & Evans, J. D. (2004). Sperm storage and antioxidative enzyme expression in the honey bee, Apis mellifera. *Insect molecular biology*, 13(2), 141-146.
12. Corby-Harris, V., & Snyder, L. A. (2018). Measuring hypopharyngeal gland acinus size in honey bee (Apis mellifera) workers. *JoVE (Journal of Visualized Experiments)*, (139), e58261.
13. Crailsheim, K. (1991). Interadult feeding of jelly in honeybee (Apis mellifera L.) colonies. *Journal of Comparative Physiology B*, *161*(1), 55-60.
14. Dainat, B., Evans, J. D., Chen, Y. P., Gauthier, L., & Neumann, P. (2012). Dead or alive: deformed wing virus and Varroa destructor reduce the life span of winter honeybees. *Appl. Environ. Microbiol.*, 78(4), 981-987.

15. Degrandi-Hoffman, G., Graham, H., Ahumada, F., Smart, M., & Ziolkowski, N. (2019). The Economics of Honey Bee (Hymenoptera: Apidae) Management and Overwintering Strategies for Colonies Used to Pollinate Almonds. J*ournal of economic entomology*, 112(6), 2524-2533.
16. Doeke, M. A., Frazier, M., & Grozinger, C. M. (2015). Overwintering honey bees: biology and management. *Current Opinion in Insect Science*, *10*, 185-193.
17. Evans, J. D. (2006). Beepath: an ordered quantitative-PCR array for exploring honey bee immunity and disease. *Journal of invertebrate pathology*, 93(2), 135-139.
18. Faber, J., & Fonseca, L. M. (2014). How sample size influences research outcomes. Dental press journal of orthodontics, 19, 27-29.
19. Fluri, P., Lüscher, M., Wille, H., & Gerig, L. (1982). Changes in weight of the pharyngeal gland and hemolymph titres of juvenile hormone, protein and vitellogenin in worker honey bees. *Journal of Insect Physiology*, *28*(1), 61-68.
20. Fukuda, H., & Sekiguchi, K. (1966). Seasonal change of the honeybee worker longevity in Sapporo, North Japan, with notes on some factors affecting the life-span. *Japanese Journal of Ecology*, *16*(5), 206-212.
21. Hartfelder, K., Bitondi, M. M., Brent, C. S., Guidugli-Lazzarini, K. R., Simões, Z. L., Stabentheiner, A., ... & Wang, Y. (2013). Standard methods for physiology and biochemistry research in Apis mellifera. *Journal of Apicultural Research*, *52*(1), 1-48.
22. Hopkins, B. K., Chakrabarti, P., Lucas, H. M., Sagili, R. R., & Sheppard, W. S. (2021). Impacts of Different Winter Storage Conditions on the Physiology of Diutinus Honey Bees (Hymenoptera: Apidae). *Journal of Economic Entomology*, *114*(1), 409-414.
23. Hrassnigg, N., & Crailsheim, K. (1998). Adaptation of hypopharyngeal gland development to the brood status of honeybee (Apis mellifera L.) colonies. *Journal of insect Physiology*, 44(10), 929-939.
24. Huang, Z. Y., & Otis, G. W. (1989). Factors determining hypopharyngeal gland activity of worker honey bees (Apis mellifera L.). *Insectes Sociaux*, *36*(4), 264-276.
25. Huang, Z. Y., Otis, G. W., & Teal, P. E. A. (1989). Nature of brood signal activating the protein synthesis of hypopharyngeal gland in honey bees, Apis mellifera (Apidae: Hymenoptera). *Apidologie*, 20(6), 455-464.
26. Huang, Z. Y., & Robinson, G. E. (1992). Honeybee colony integration: worker-worker interactions mediate hormonally regulated plasticity in division of labor. *Proceedings of the National Academy of Sciences*, *89*(24), 11726-11729.
27. Huang Z-Y, Robinson GE, Borst DW (1994) Physiological correlates of division of labor among similarly aged honey bees. *J Comp Physiol A* 174:731 739.
28. Huang, Z. Y., & Robinson, G. E. (1995). Seasonal changes in juvenile hormone titers and rates of biosynthesis in honey bees. *Journal of Comparative Physiology B*, 165(1), 18-28.
29. Ifantidis, M. D. (1983). Ontogenesis of the mite Varroa jacobsoni in worker and drone honeybee brood cells. *Journal of Apicultural Research*, *22*(3), 200-206.

30. Jeffree, E. P. (1956). Winter brood and pollen in honeybee colonies. *Insectes sociaux*, *3*(3), 417-422.
31. Kunc, M., Dobeš, P., Hurychová, J., Vojtek, L., Poiani, S. B., Danihlík, J., ... & Hyršl, P. (2019). The Year of the Honey Bee (Apis mellifera L.) with Respect to Its Physiology and Immunity: A Search for Biochemical Markers of Longevity. *Insects*, *10*(8), 244.
32. Le Conte, Y., & Navajas, M. (2008). Climate change: impact on honey bee populations and diseases. *Revue Scientifique et Technique-Office International des Epizooties*, *27*(2), 499-510.
33. Leoncini, I., Le Conte, Y., Costagliola, G., Plettner, E., Toth, A. L., Wang, M., ... & Robinson, G. E. (2004). Regulation of behavioral maturation by a primer pheromone produced by adult worker honey bees. *Proceedings of the National Academy of Sciences,* 101(50), 17559-17564.
34. Lin, Y. W., Chen, C. H., & Hsu, C. Y. (2022). Middle-Aged Worker Bees Express Higher Innate Immunity than Young Worker Bees in the Abdomen without the Digestive Tract of Worker Bees Reared in an Incubator. Insects, 13(2), 209.
35. Malka, O., Shnieor, S., Hefetz, A., & Katzav-Gozansky, T. (2007). Reversible royalty in worker honeybees (Apis mellifera) under the queen influence. *Behavioral Ecology and Sociobiology*, 61(3), 465-473.
36. Mattila, H. R., Harris, J. L., & Otis, G. W. (2001). Timing of production of winter bees in honey bee (Apis mellifera) colonies. *Insectes Sociaux*, *48*(2), 88-93.
37. Mattila, H. R., & Otis, G. W. (2007). Dwindling pollen resources trigger the transition to broodless populations of long-lived honeybees each autumn. *Ecological Entomology*, *32*(5), 496-505.
38. Maurizio, A., & Hodges, F. E. D. (1950). The influence of pollen feeding and brood rearing on the length of life and physiological condition of the honeybee preliminary report. *Bee World*, *31*(2), 9-12.
39. Mohammedi, A., Crauser, D., Paris, A., & Le, Y. C. (1996). Effect of a brood pheromone on honeybee hypopharyngeal glands. Comptes rendus de l'Academie des sciences. *Serie III, Sciences de la vie*, 319(9), 769-772.
40. Muenz, T. S., Maisonnasse, A., Plettner, E., Le Conte, Y., & Rössler, W. (2012). Sensory reception of the primer pheromone ethyl oleate. *Naturwissenschaften*, *99*(5), 421-425.
41. Nguyen, B. K., Ribière, M., vanEngelsdorp, D., Snoeck, C., Saegerman, C., Kalkstein, A. L., & Haubruge, E. (2011). Effects of honey bee virus prevalence, Varroa destructor load and queen condition on honey bee colony survival over the winter in Belgium. *Journal of Apicultural Research*, *50*(3), 195-202.
42. Page Jr, R. E., Robinson, G. E., Britton, D. S., & Fondrk, M. K. (1992). Genotypic variability for rates of behavioral development in worker honeybees (Apis mellifera L.). *Behavioral Ecology*, 3(2), 173-180.

43. Pankiw, T., Sagili, R. R., & Metz, B. N. (2008). Brood pheromone effects on colony protein supplement consumption and growth in the honey bee (Hymenoptera: Apidae) in a subtropical winter climate. *Journal of economic entomology*, *101*(6), 1749-1755.
44. Peters, L., Zhu-Salzman, K., & Pankiw, T. (2010). Effect of primer pheromones and pollen diet on the food producing glands of worker honey bees (Apis mellifera L.). *Journal of insect physiology*, *56*(2), 132-137.
45. Rangel, J., Böröczky, K., Schal, C., & Tarpy, D. R. (2016). Honey bee (Apis mellifera) queen reproductive potential affects queen mandibular gland pheromone composition and worker retinue response. *PloS one*, 11(6), e0156027.
46. Robinson, G. E. (1987). Regulation of honey bee age polyethism by juvenile hormone. *Behavioral ecology and sociobiology*, *20*(5), 329-338.
47. Robinson, G. E., & Ratnieks, F. L. (1987). Induction of premature honey bee (Hymenoptera: Apidae) flight by juvenile hormone analogs administered orally or topically. *Journal of Economic Entomology*, *80*(4), 784-787.
48. Robinson, G. E., Page Jr, R. E., Strambi, C., & Strambi, A. (1992). Colony integration in honey bees: mechanisms of behavioral reversion. *Ethology*, *90*(4), 336-348.
49. Ruijter, J. M., Pfaffl, M. W., Zhao, S., Spiess, A. N., Boggy, G., Blom, J., ... & Vandesompele, J. (2013). Evaluation of qPCR curve analysis methods for reliable biomarker discovery: bias, resolution, precision, and implications. Methods, 59(1), 32-46.
50. Sagili, R. R., & Pankiw, T. (2009). Effects of brood pheromone modulated brood rearing behaviors on honey bee (Apis mellifera L.) colony growth. *Journal of insect behavior*, *22*(5), 339-349.
51. Schmickl, T., & Crailsheim, K. (2001). Cannibalism and early capping: strategy of honeybee colonies in times of experimental pollen shortages. *Journal of Comparative Physiology A*, *187*(7), 541-547.
52. Seeley, T. D., & Visscher, P. K. (1985). Survival of honeybees in cold climates: the critical timing of colony growth and reproduction. *Ecological Entomology*, *10*(1), 81-88.
53. Seeley, T. D. (1989). The honey bee colony as a superorganism. *American Scientist*, *77*(6), 546-553.
54. Seeley, T. D., & Kolmes, S. A. (1991). Age polyethism for hive duties in honey bees—illusion or reality?. *Ethology*, *87*(3-4), 284-297.
55. Shehata, S. M., Townsend, G. F., & Shuel, R. W. (1981). Seasonal physiological changes in queen and worker honeybees. *Journal of Apicultural Research*, *20*(2), 69-78.
56. Smedal, B., Brynem, M., Kreibich, C. D., & Amdam, G. V. (2009). Brood pheromone suppresses physiology of extreme longevity in honeybees (Apis mellifera). J*ournal of Experimental Biology*, 212(23), 3795-3801.
57. Stabentheiner, A., Pressl, H., Papst, T., Hrassnigg, N., & Crailsheim, K. (2003). Endothermic heat production in honeybee winter clusters. *Journal of Experimental Biology*, *206*(2), 353-358.

58. Standifer, L. N. (1967). A comparison of the protein quality of pollens for growth-stimulation of the hypopharyngeal glands and longevity of honey bees, Apis mellifera L.(Hymenoptera: Apidae). *Insectes Sociaux*, *14*(4), 415-425.
59. Tsvetkov, N., Cook, C. N., & Zayed, A. (2019). Effects of group size on learning and memory in the honey bee Apis mellifera. *Journal of Experimental Biology*, 222(9), jeb193888.
60. van Dooremalen, C., Gerritsen, L., Cornelissen, B., van der Steen, J. J., van Langevelde, F., & Blacquière, T. (2012). Winter survival of individual honey bees varroa and honey bee colonies depends on level of Varroa destructor infestation. *PloS one*, *7*(4), e36285.
61. Winston, M. L. (1980). Seasonal patterns of brood rearing and worker longevity in colonies of the Africanized honey bee (Hymenoptera: Apidae) in South America. *Journal of the Kansas Entomological Society*, 157-165.
62. Winston ML. The Biology of the Honey Bee. Cambridge, Massachusetts: Harvard University Press; 1987. 60 p.
63. Yang X., Cox-Foster D.L. Impact of an ectoparasite on the immunity and pathology of an invertebrate: Evidence for host immunosuppression and viral amplification. P*roc. Natl. Acad. Sci.* USA. 2005;21:7470–7475. doi: 10.1073/pnas.0501860102.
64. Zhao, S., & Fernald, R. D. (2005). Comprehensive algorithm for quantitative real-time polymerase chain reaction. Cell Reports, 35(4).